ECOGEOGRAPHIC, ADAPTIVE, AND PHYLOGENETIC VARIATION IN THE CRESTED DUCK (*LOPHONETTA SPECULARIOIDES*) AND THEIR HEMOGLOBINS IN THE ANDES

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ECOGEOGRAPHIC, ADAPTIVE, AND PHYLOGENETIC VARIATION IN THE CRESTED DUCK (*LOPHONETTA SPECULARIOIDES*) AND THEIR HEMOGLOBINS IN THE ANDES

A

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Abstract

Tolerance to high-altitude hypoxia in animals varies widely and is a key factor in determining survival at high elevation. The Andean Cordillera of South America, which spans large elevational and latitudinal gradients, enables the study of native highland populations and the characteristics of hemoglobin proteins that are locally adapted for high-altitude respiration. The waterfowl populations of South America are understudied, little data on demographics and behavior are currently available, and only recently have they been investigated using molecular tools. We studied population genetics, phylogeography, and ecogeographic variation in the crested duck (*Lophonetta specularioides*). The crested duck is a dabbling duck, and it comprises two subspecies endemic to highland and lowland regions of South America. The primary objective of this study was to investigate the genetic differentiation between highland and lowlands populations of crested ducks using molecular markers with varying modes of inheritance and rates of substitution. The second objective was to evaluate morphological differences between the subspecies to better understand the forces shaping morphology in the two different environments. A third objective was to provide additional information on the taxonomic relationships and natural history of the crested duck.

First, we examined the population genetics of the three adult hemoglobins (αD, αA, βA), six autosomal introns, and mtDNA. This multi-locus analysis revealed a significant pattern of differentiation between highland and lowland populations. Four hemoglobin amino acid replacements were found in crested duck that may play a role in influencing high-altitude respiration. The lack of evidence for gene flow for hemoglobin

alleles between highland and lowland populations and the biochemical properties of the amino acid substitutions themselves are consistent with the effects of selection acting on these loci. Overall body size was larger for the highland subspecies, body size was intermediate in mid-elevation environments, and smaller individuals were found in the lowlands of Patagonia. We also performed a multi-locus phylogenetic analysis to determine the relationships of *Lophonetta* within the South American duck clade. Finally, we determined the proportion of genes expressed in bone marow of adult crested duck finding mostly genes related to hemopoietic and immune function.

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General Introduction

Tolerance to high-altitude hypoxia varies widely, and some organisms have evolved extraordinary capabilities to survive under limited oxygen availability (Hochachka and Lutz 2001). Hypoxia is among the most important ecological factors affecting survival in high-altitude regions, typically defined as elevations above 2,000 m (Hornbein and Schoene 2001). The partial pressure of oxygen (P_{O2}) decreases with altitude by approximately ten percent per thousand meters. At elevations such as 4,000 m in the Andean or Himalayan plateaus the P_{O2} of inspired air is 60% of that at sea level (Beall 2007). Highland organisms have developed a variety of physiological and molecular mechanisms to cope with life at high elevations (Hochachka and Somero 2002, Weber 2007, Storz and Moriyama 2008). Several recent studies have shown that genetically based adaptations in hemoglobins, which bind oxygen in the lungs and deliver it to the respiring tissues (Hardison 2001), have played an important role in physiological adaptation to high-altitude hypoxia (Storz et al. 2007, Weber 2007, Storz and Moriyama 2008).

Waterfowl inhabiting South America offer an excellent opportunity to study high-elevation adaptation because they are distributed in the alpine wetlands and grasslands of the Andean Cordillera, which span large elevational and latitudinal gradients. In this study, we assessed population genetic structure and evaluated evidence for local adaptation in the hemoglobin genes of the crested duck (*Lophonetta specularioides*), a monotypic genus of dabbling duck (Anatinae) endemic to the central Andean and Patagonian regions of South America. Crested duck comprises two subspecies:

Patagonian crested duck (*L. s. specularioides*) and Andean crested duck (*L. s. alticola*; Phillips 1922–1926, Johnsgard 1978). The two subspecies inhabit different elevational environments ranging from 5,000 meters in the central high Andes (*L. s. alticola*) to sea level in Patagonia and the Malvinas/Falkland Islands (*L. s. specularioides*). Crested ducks are thus an excellent species for investigating how changes in the amino acid sequence of the adult αA, αD, and βA hemoglobin genes might confer resistance to hypoxia in different elevational zones.

Despite the importance of mountain regions as biodiversity hotspots (Lomolino 2001, Körner 2002), and the fact that the Andean Cordillera has a higher diversity of plant and animal life than any other part of the world (Fjeldså and Krabbe 1990), little information exists on Andean waterfowl in general, and crested ducks in particular. There has been no previous population genetics work conducted for any South American duck species. Also, to our knowledge, this is the first extensive study to evaluate genetic relationships between South America duck genera using molecular tools.

The specific objectives of this research were to:

1. Study the phylogenetic relationships of the four South American duck genera: *Amazonetta*, *Lophonetta*, *Speculanas*, and *Tachyeres*; and further test the monophyly of this clade.
2. Evaluate the morphological variation in the two subspecies of crested duck to better understand the forces acting to shape geographic variation in morphology of highland and lowland populations.

3. Identify the genes expressed in the bone marrow (the site of hemoglobin synthesis) of crested ducks inhabiting high-altitude regions and compare them to previous studies of high-altitude gene expression.
4. Assess the population genetic structure of South American crested ducks using a multi-locus approach and coalescent analysis, and evaluate evidence for selection on the hemoglobin genes using a series of interlocus contrasts.

Chapter 1:

Phylogenetic relationships of *Amazonetta*, *Speculanas*, *Lophonetta*, and *Tachyeres*: four morphologically divergent duck genera endemic to South America[1]

1.1 Abstract

We studied the phylogenetic relationships of four duck genera endemic to South America: Brazilian teal (*Amazonetta brasiliensis*), spectacled duck (*Speculanas specularis*), crested duck (*Lophonetta specularioides*), and four species of steamer ducks (*Tachyeres patachonicus*, *T. leucocephalus*, *T. pteneres*, *T. brachypterus*). Genetic divergence within and among species was compared using population-level sampling of the mitochondrial DNA (mtDNA) control region, supplemented with three additional mtDNA genes and six independent nuclear loci from one individual of each species and a variety of outgroup taxa. The monophyly of these four morphologically divergent South American genera was strongly supported. Within this clade, *Amazonetta* and *Speculanas* were supported as sister species in all analyses, but different gene regions yielded conflicting or ambiguous results for *Lophonetta* and *Tachyeres*. This lack of resolution resulted from little informative variation in nuclear loci and high levels of homoplasy in the mtDNA control region. Control region sequences from the four *Tachyeres* species fell

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[1] Bulgarella, M., M.D. Sorenson, J.L. Peters, R.E. Wilson, and K.G. McCracken. 2010. Phylogenetic relationships of *Amazonetta*, *Speculanas*, *Lophonetta*, and *Tachyeres*: four morphologically divergent duck genera endemic to South America. *Journal of Avian Biology* 41: 186–199.

into two distinct clades. In one clade, *T. patachonicus* and *T. leucocephalus* share a set of closely related haplotypes ($\leq$ 0.6 % sequence divergence); while no identical haplotypes were shared between species, the control region phylogeny was insufficiently resolved to either support or reject reciprocal monophyly. The second clade, ~1.7% divergent from the first, comprised haplotypes of the Falkland Islands species *T. brachypterus* and a captive individual of *T. pteneres*. These distinctive South American ducks likely experienced two bouts of rapid diversification, thus making analysis of their phylogenetic relationships difficult. Incomplete lineage sorting, founder effects, and perhaps introgression likely have contributed to obscuring the relationships among steamer ducks.

1.2 Introduction

Multiple independent loci are often required to confidently resolve species-level phylogenies, but different genes may support different relationships among taxa. Several phenomena can lead to incongruent gene trees. The topology of a given gene tree may differ from that of the species tree because of evolutionary rate heterogeneity, convergent base composition bias, stochastic lineage sorting, introgressive hybridization, or simple sampling error in the presence of homoplasy (Funk and Omland 2003, McCracken and Sorenson 2005, Avise 2007). These obstacles to phylogenetic reconstruction are exacerbated when taxa diverge rapidly, potentially leading to an unresolved polytomy among three or more taxa (Hoelzer and Melnick 1994). In some cases, a polytomy can be resolved by analyzing additional DNA sequence data or different types of character data (i.e., a soft polytomy). In contrast, a hard polytomy, resistant to additional data and analyses, may reflect the nearly simultaneous divergence of three or more lineages (Hoelzer and Melnick 1994).

Improving phylogenetic resolution typically relies on collecting more DNA sequence data from multiple loci, but phylogeneticists face a difficult empirical problem. Mitochondrial DNA (mtDNA) seems ideal for species-level phylogenies, given its high mutation rate, low effective population size, and lack of recombination (Avise et al. 1987, Moritz et al. 1987, Moore 1995). Like any other single locus, however, mtDNA provides only one estimate of the species tree (Avise 2006), and the probability that it accurately reflects the species tree may be low if the time between successive speciation events is short (Nei 1987, Pamilo and Nei 1988, Wu 1991). Thus, one would like to infer

phylogenies based on data from several independent loci (Pamilo and Nei 1988, Wu 1991, Peters et al. 2005), but the only source of additional loci, the nuclear genome, offers none of the advantages of mtDNA. Nuclear gene trees may be poorly resolved due to lower mutation rates and are substantially less likely than mtDNA to track the species tree through a short internode (Moore 1995). In addition to the stochasticity of mutation and lineage sorting, individual gene trees may be subjected to the misleading effects of introgressive hybridization, making it essential to consider multiple independent loci (Page and Charleston 1998).

1.2.1 South American duck genera

Several unresolved questions about the phylogenetic relationships of dabbling ducks (tribe Anatini) persist despite several comprehensive morphological and molecular analyses (Livezey 1986a, 1991, Johnson and Sorenson 1998, 1999). Livezey (1991) published the first modern dabbling duck phylogeny, based on cladistic analysis of 120 morphological characters. He included all dabbling ducks and many of the "perching ducks" in the tribe Anatini, classifying *Anas* and the genera *Mareca* (comprising six species more often included in *Anas*), *Amazonetta, Callonetta, Lophonetta*, and *Speculanas* within the subtribe Anateae. However, molecular analyses based on the mitochondrial ND2 and cytochrome *b* genes (Johnson and Sorenson 1998, 1999) supported the monophyly of a "dabbling duck" clade comprising all *Anas* species (including *Mareca*) and four additional genera endemic to South America: Brazilian teal (*Amazonetta brasiliensis*), crested duck (*Lophonetta specularioides*), spectacled duck

(*Speculanas specularis*), and steamer ducks (*Tachyeres* spp.). Molecular data also provided strong support for the monophyly of these four South American genera, but left basal relationships within this clade and the position of this South American clade in relation to three other *Anas* clades poorly resolved (Johnson and Sorenson 1999). These findings are noteworthy because Livezey (1986a, 1996) grouped steamer ducks, together with torrent ducks (*Merganetta*), and blue duck (*Hymenolaimus*) in a clade near the shelducks and sheldgeese (subfamily Tadorninae). *Amazonetta* was grouped with *Callonetta* outside the Anatini, whereas *Lophonetta* and *Speculanas* were placed as a sister clade to *Anas* dabbling ducks (Livezey 1991). The shared ancestry of *Amazonetta*, *Speculanas*, *Lophonetta*, and *Tachyeres* had thus not been previously recognized before Johnson and Sorenson's studies (1998, 1999), and phylogenetic relationships among these genera have received little study to date.

Brazilian teal is found in tropical wetlands of northeastern South America (Narosky and Yzurieta 2003). Spectacled duck is restricted to freshwater lakes, ponds and rivers of the southern Andes in Patagonia, north to southern Mendoza (Harris 1998). Crested duck is widely distributed in the Andes from Perú south to Tierra del Fuego, throughout coastal and steppe regions of southern Patagonia, and east to the Falkland Islands (Kear 2005). In contrast to spectacled duck, which predominantly occurs throughout the forested regions of the Patagonian Andes, crested ducks inhabit the treeless steppe regions of Patagonia. *Tachyeres* includes four species. The flying steamer duck (*Tachyeres patachonicus*) inhabits freshwater and marine habitats throughout southern Argentina, Chile, and the Falkland Islands. The other three species are flightless.

The flightless steamer duck (*T. pteneres*) is endemic to coastal habitats of Tierra del Fuego and southern Chile. The white-headed steamer duck (*T. leucocephalus*) is endemic to the coast of Chubut, Argentina. The Falkland steamer duck (*T. brachypterus*) is endemic to the Falkland Islands (Fig. 1.1). Livezey (1986b) and Corbin et al. (1988), using morphological and allozyme data, respectively, concluded that *T. patachonicus* is the sister species to a clade comprising the three flightless species, and that *T. leucocephalus* and *T. brachypterus* are each other's closest relatives.

To further test the monophyly of this clade of South American ducks and to resolve relationships among the four genera as well as the species of steamer ducks, we completed phylogenetic analyses at two different hierarchical levels. First, we sequenced the mtDNA control region from multiple individuals of each of the four genera and seven species to compare genetic divergence within and between species, and specifically test whether a lack of reciprocal monophyly contributes to poorly resolved relationships among the four species of steamer ducks. Second, to better resolve relationships among genera, we sequenced three additional mtDNA genes and six independent nuclear loci from one individual of each of the South American species and a variety of outgroup taxa included in previous morphological analyses (Livezey 1986a, 1991, 1996).

1.3 Materials and methods

1.3.1 Sampling, PCR, and DNA sequencing

We collected Brazilian teal (n = 7), spectacled ducks (n = 2), crested ducks (n = 23), and steamer ducks (*T. patachonicus* n = 7, *T. leucocephalus* n = 3, *T. brachypterus* n = 4) in

Argentina and the Falkland Islands between 2001 and 2003 (Appendix 1.1). The identifications of all coastal specimens of *T. patachonicus* and *T. lecucocephalus* were verified with wing-loading criteria published by Humphrey and Livezey (1982), as shown in Table 1 of Wilson et al. (2007). One *T. pteneres* sample was obtained from a captive bird in the United States, and the other from a bird on Navarino Island, Chile. Five representative dabbling ducks (*Anas crecca crecca*, *A. c. carolinensis*, *A. acuta*, *A.americana*, *A. clypeata*) and 18 other waterfowl genera shown in Appendix 1.1 were selected as outgroups.

DNA sequences were obtained using standard protocols for DNA extraction, PCR, and sequencing (e.g., McCracken and Sorenson 2005). We amplified and sequenced most of the mtDNA control region, tRNA-Phe, and the 5' end of the 12S rRNA gene (corresponding to bp 82 to 1529 in the chicken [*Gallus gallus*] mitochondrial genome; Desjardins and Morais 1990) from each of the 53 sampled *Amazonetta*, *Speculanas*, *Lophonetta*, and *Tachyeres* specimens. We also sequenced 12S rRNA (12S), NADH dehydrogenase subunit 2 (ND2), part of ND5, cytochrome *b*, and adjacent t-RNA genes (chicken mtDNA genome positions 1268 to 2293, 5217 to 6312, and 14 771 to 16 063; Desjardins and Morais 1990) from one individual of each species included in the control region data set and each of the outgroup species. Each region was amplified and sequenced using two or more overlapping primer pairs (Appendix 1.2). Finally, we sequenced six nuclear loci located on five different chromosomal linkage groups in the chicken genome (Hillier et al. 2004): the complete coding region and intron sequences of the αA and βA hemoglobin subunits (HBA2, HBB, respectively), lecithin-cholesterol

acyltransferase introns 2, 3 and 4 (LCAT), T-cell surface glycoprotein CD4 precursor intron 4 (CD4), and phosphoenolpyruvate carboxykinase introns 3 and 9 (PCK1). The HBA and HBB sequences each included three exons and two introns; LCAT included two complete exons and three introns; the other nuclear sequences were primarily introns with limited flanking exons sequences. We designed primers for each locus (Appendix 1.2) based on GenBank sequences for chicken and other vertebrates. Sequences from opposite strands were reconciled and verified for accuracy using Sequencher 4.7 (Gene Codes, Ann Arbor, Michigan). Sequences are archived in GenBank (see Appendix 1.1).

1.3.2 Phylogenetic analysis of the mtDNA control region

The mtDNA control region sequences varied in length due to insertions and deletions of nucleotides (Baker and Marshall 1997). We aligned control region sequences for the four South American genera and five representative species of *Anas* dabbling ducks using direct optimization as implemented in POY 3.0.11 (Wheeler et al. 2003). The analysis included all 53 control region haplotypes and used 10 random addition replicates (each limited to five trees), equal weights for all changes, tree bisection and reconnection (TBR) branch-swapping, and an insertion-deletion cost equal to one. The resulting implied alignment was used for subsequent analyses.

We used AIC (Akaike 1973) as implemented in Modeltest 3.7 (Posada and Crandall 1998) to determine the model of sequence evolution that best fit the mtDNA control region data. With these parameter values fixed, we completed 100 replicate maximum likelihood searches in PAUP* 4.0b10 (Swofford 2002), each with random

addition of taxa. We also used equal weights parsimony and 1000 heuristic tree searches to find all equally parsimonious trees for the optimized alignment. Multiple base indels were coded as missing data, and new binary characters for each unique gap (0 = absent, 1 = present) were added to the end of the data matrix. Single-base indels were included in the parsimony analysis by treating gaps as a fifth character state. We identified mtDNA control region characters supporting alternative branching patterns among the four South American duck genera, and compared these characters in terms of sequence position, substitution types, number of steps, and consistency indices. Finally, trees with alternative basal relationships found in maximum likelihood and parsimony analyses, respectively, were compared under both optimality criteria using a Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999).

1.3.3 Additional mtDNA data

Additional mtDNA sequence data from three gene regions (12S rRNA, ND2, and ND5/cytochrome *b*) for a single representative of each species were assembled with the control region data. The combined mtDNA data set comprised 4468 characters. We conducted maximum likelihood and Bayesian analyses on the combined mitochondrial data set and individual data partitions using a single, best-fit model of sequence evolution for each partition. Data were partitioned by gene region (CR, 12S, ND2, ND5/cytochrome *b*) or by codon position, combining data for ND2 and ND5/cytochrome *b* (Table 1.3). We also evaluated two mixed models with separate parameters estimated for each partition. Mixed model 1 partitioned the data into gene regions: CR, 12S, ND2,

and ND5/cytochrome *b*, whereas mixed model 2 included five data partitions: CR, 12S, and 1st, 2nd, and 3rd codon positions combined across ND2 and ND5/cytochrome *b*. Best-fit models were assessed using AIC, and alternative topologies were compared using the approximately unbiased test of Shimodaira (2002) (Table 1.3).

Clade probabilities for the combined mtDNA data were obtained from the posterior distribution using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2005). Bayesian analyses were replicated twice, each with four Markov chains of 2.5 million generations. Trees were sampled every 1000 generations, of which the first 0.5 million generations were discarded as burnin.

1.3.4 Analysis of nuclear DNA

Alignments for each of the six nuclear gene regions were unambiguous. Multiple base indels were coded as missing data, and new binary characters for each unique gap (0 = absent, 1 = present) were added to the end of the data matrix. Double peaks in nuclear sequences, reflecting heterozygous positions, were coded with IUPAC degeneracy codes and treated as polymorphisms. We performed 1000 heuristic tree searches using parsimony with random addition of taxa to find the most parsimonious tree(s) for each nuclear locus.

We also concatenated the six nuclear loci and conducted maximum likelihood analysis using the best fit model of sequence evolution for the entire nuclear data set, as selected by AIC. Statistical support for clades was evaluated by nonparametric bootstrapping (Felsenstein 1985) in PAUP* 4.0b10 (Swofford 2002). We completed full

heuristic searches for 1000 or 100 pseudoreplicate data sets for parsimony and maximum likelihood analyses, respectively. Trees were rooted with *Anas americana*, *A. c. crecca*, *A. c. carolinensis*, *A. acuta*, and *A. clypeata* as a paraphyletic outgroup to the South American clade, based on the results of Johnson and Sorenson (1998, 1999) as well as additional analyses presented below.

To more thoroughly test the monophyly of the four South American genera, we conducted a Bayesian phylogenetic analysis of eight concatenated mtDNA and nuclear loci for one individual each of *Amazonetta*, *Speculanas*, *Lophonetta*, *Tachyeres*, five representative *Anas* dabbling duck species, and 18 other waterfowl genera. Three mtDNA genes (ND2, 12S, cytochrome *b*) and five nuclear loci (HBA2, LCAT, CD4, PCK1-3, and PCK1-9) were used for this analysis, and the alignment for each locus was made by eye. One true goose and a swan (subfamily Anserinae) were included as outgroups. Clade probabilities for the combined data set were obtained from the posterior distribution with MrBayes 3.1.2, using the same Markov chain protocols described above and the GTR+I+G model, as determined in Modeltest 3.7. Support for each node was also measured using nonparametric boostrapping (Felsenstein 1985), with equal weights parsimony and full heuristic tree searches for 1000 pseudoreplicate data sets using PAUP* 4.0b10.

1.4 Results

1.4.1 Monophyly of the South American duck genera

The monophyly of the four South American duck genera and their relationship to dabbling ducks were both strongly supported in analyses with an expanded set of outgroup taxa. Bayesian posterior probability and maximum parsimony bootstrap support for a clade comprising *Amazonetta*, *Speculanas*, *Lophonetta*, *Tachyeres* were 100%, and equally strong support also was observed for a clade comprising these four South American genera and *Anas* (Fig. 1.2). There was no evidence that *Amazonetta*, *Speculanas*, *Lophonetta*, or *Tachyeres* are closely related to any other waterfowl genera outside *Anas*. Therefore, the selection of five representative *Anas* species as the outgroup for all subsequent analyses was fully justified.

1.4.2 mtDNA control region

The mtDNA control region alignment comprised 1314 characters, of which 169 (13%) were variable and 157 (12%) were parsimony informative (Table 1.1). An 11 bp deletion at positions 879–889 was a synapomorphy for *Tachyeres*. Optimization alignment and unweighted parsimony analysis of 53 sequences produced 36 equally parsimonious trees (length = 489, CI = 0.681). Genus-level relationships were (*Tachyeres* (*Lophonetta* (*Amazonetta, Speculanas*))) (Fig. 1.3). Four trees with equal likelihood (–lnL = 3905.07) were obtained in the ML analysis, all with identical relationships among genera: ((*Tachyeres, Lophonetta*) (*Amazonetta, Speculanas*)). The best-fit model was TVM+I+G (Fig. 1.3). While a sister relationship between *Amazonetta* and *Speculanas* was supported

in both analyses, parsimony and maximum likelihood produced conflicting results for basal relationships in the South American clade. In the ML analysis, *Lophonetta* and *Tachyeres* were sister taxa, albeit with only moderate bootstrap support (80%). In contrast, the parsimony analysis indicated moderate support (90% bootstrap value) for *Lophonetta* as the sister species to *Amazonetta-Speculanas*.

These alternative topologies, however, did not differ significantly under either parsimony or maximum likelihood criteria. Table 1.2 lists individual control region characters that support each of the two resolutions of basal relationships in the South American clade. Ten characters (8 transitions and 2 indels) with a mean CI of 0.78 have fewer steps on the maximum parsimony topology in which *Lophonetta* is sister to *Amazonetta-Speculanas*, whereas constraining the analysis to find a tree with *Lophonetta* sister to *Tachyeres* (the maximum likelihood topology) results in fewer steps for only 5 characters (4 transitions and 1 transversion), but with a slightly higher overall mean CI (0.87). Likelihood scores associated with these two alternative topologies did not differ significantly using a Shimodaira-Hasegawa test ($P = 0.19$, Shimodaira and Hasegawa 1999).

1.4.3 Relationships within *Tachyeres*

All of the *Tachyeres* we examined fell into one of two distinct mtDNA clades with sequence divergence between clades of 1.4 to 2.1%. One clade included all *T. leucocephalus* and *T. patachonicus* individuals plus a *T. pteneres* from Navarino Island, Chile. The second clade included all four *T. brachypterus* from the Falkland Islands and a

second *T. pteneres* obtained from a private avicultural collection. Although none of the *Tachyeres* species were strictly monophyletic, it is interesting to note that no identical haplotypes were shared between species (Fig. 1.3). Unfortunately, we cannot rule out the possibility that one or both of the *T. pteneres* used in our analysis were from misidentified individuals; the complete history of the captive individual is not known and no voucher specimen is available for the sample from Navarino Island, Chile, which lies within a region where *T. patachonicus* and *T. pteneres* are broadly sympatric.

1.4.4 Combined analyses of additional mtDNA

Sequence variation in ND2 and ND5/cytyochrome *b* was comparable to the control region in terms of number of informative and variable sites, but 12S rRNA had lower levels of variation (Table 1.1). Consistency and rescaled consistency indices were similar for the four mtDNA gene regions, although ND2 showed slightly higher values for both indices (Table 1.1).

The combined mtDNA data set was 4468 characters, of which 460 (10.3%) were variable and 233 (5.2%) were parsimony informative. A single most parsimonious tree was obtained (length = 1343, CI = 0.675). This combined data set yielded the same grouping of the taxa as in the parsimony analysis of control region data only, albeit with higher bootstrap support at most nodes (Fig. 1.4A, Table 3). *Lophonetta* was supported as the sister species to *Amazonetta-Speculanas* in the parsimony tree with moderate bootstrap support (88%, Fig. 1.4A). The most likely tree for the combined mtDNA data set (–lnL = 12 040.71) supported a sister relationship between *Lophonetta* and *Tachyeres*

with weak bootstrap support (60%) and low posterior probability (0.24, Fig. 1.4B). Similar to results from the control region, maximum likelihood and parsimony produced different relationships for *Lophonetta* and *Tachyeres* despite including additional mtDNA data in the analysis. Likelihood scores for the two alternative topologies were not significantly different ($P = 0.25$; Shimodaira-Hasegawa test).

Mixed model analyses with parameters estimated independently for the different mtDNA data partitions provided a better fit as measured by AIC but produced the same phylogenetic inferences as single-model ML and Bayesian analyses. Notably, excluding control region sequences from the analysis resulted in the same grouping of taxa in both maximum likelihood and parsimony analyses (*Tachyeres* (*Lophonetta* (*Amazonetta*, *Speculanas*))) (figure not shown).

1.4.5 Nuclear DNA data

Concatenating the six nuclear loci resulted in 5559 characters, of which 107 (2%) were variable but only 33 (< 1%) were parsimony informative. Each locus had from one to twelve informative characters: HBA2, nine; HBB, five; LCAT, twelve; CD4, two; PCK1-3, one; PCK1-9, four.

Parsimony analysis of the six concatenated sequences produced two equally parsimonious trees (length = 640, CI = 0.959). Both of these trees included *Lophonetta* as the sister group to the other three genera in the South American clade (*Lophonetta* (*Tachyeres* (*Amazonetta*, *Speculanas*))), although this resolution of basal relationships was weakly supported (Fig. 1.4C). The model of sequence evolution for the concatenated

nuclear loci was HKY+I. Two equally likely trees (–lnL = 9466.43; Fig. 1.4D) supported the same relationships (*Tachyeres* (*Lophonetta* (*Amazonetta*, *Speculanas*))) as found in all parsimony analyses of the mtDNA data, and in maximum likelihood analyses of three mtDNA genes (i.e., excluding the control region), albeit with weak bootstrap support (53%). There was no difference in likelihood scores between the two possible topologies as determined by the Shimodaira-Hasegawa test ($P = 1.00$).

1.5 Discussion

Rapid cladogenesis generates phylogenies with short internal branches, thus limiting the accumulation of informative genetic variation needed to resolve phylogenetic relationships (Hoelzer and Melnick 1994, Rokas and Carroll 2006). Even with somewhat longer internodes, relationships may be obscured by the stochastic effects of homoplasy and lineage sorting. Finally, a broadly distributed ancestral species may give rise to two or more new species through founder events or peripatric speciation, without going extinct, thus yielding non-dichotomous phylogenetic patterns. All of these processes may have contributed to the difficulty of resolving relationships in our study.

Johnson and Sorenson (1999) found that *Amazonetta, Speculanas, Lophonetta,* and *Tachyeres* formed a strongly supported clade, and our analysis of additional loci and outgroup taxa strongly corroborates this finding. However, relationships within this well-supported clade were not well resolved. In our study, both mtDNA and nuclear DNA supported *Amazonetta* and *Speculanas* as sister-species, but the relationships of *Lophonetta* and *Tachyeres* were less certain. Three different resolutions of a basal

trichotomy were found depending on the data set (mtDNA versus nuclear) and method of analysis (parsimony versus maximum likelihood). However, both parsimony analysis of mtDNA and maximum likelihood analysis of nuclear sequences placed *Lophonetta* as the sister group of *Amazonetta-Speculanas*. In contrast, maximum likelihood analysis of the mtDNA data placed *Lophonetta* sister to the morphologically divergent *Tachyeres*, although this result was obtained only when control region data were included. Sequence data from the six nuclear loci contributed relatively little additional phylogenetic information, such that combined analysis of both mtDNA and nuclear data yielded results similar to that for mtDNA alone. For most nuclear loci, gene trees were poorly resolved or unresolved due to a general lack of informative variation, owing to their slower evolutionary rates. This contrast in evolutionary rates between mtDNA and nuclear loci is a general challenge for phylogeneticists seeking to test species level relationships with multiple loci and suggests the need for larger data sets and new methods of analysis (e.g., Maddison and Knowles 2006, Edwards et al. 2007).

Uncertain basal relationships among the four South American genera primarily reflect the difficulty of resolving a short internal node in the face of stochastic effects (e.g., homoplasy in mtDNA, limited informative variation in nuclear loci), rather than any significant conflict in our data set. Different topologies found in parsimony and maximum likelihood analyses of the control region data, for example, reflect the relative influence of a small number of characters. Two transversions uniting *Lophonetta* and *Tachyeres* at positions 259 (Table 1.2) and 269 (where *Lophonetta* has T, *Tachyeres* has C, and other taxa have A) strongly influence the likelihood analysis, whereas two indels

that help unite *Lophonetta* with *Amazonetta* and *Speculanas* in the parsimony analysis (Table 1.2) are effectively ignored because gaps are treated as missing data in the likelihood analysis. All four of these characters lie within highly variable regions with numerous insertions and deletions across dabbling ducks. Peters et al. (2005) encountered a similar discrepancy when analyzing control region data in wigeons and their allies (*Anas* spp.); the most likely topology for the control region disagreed with the placement of *Anas penelope* in other analyses. Taken together, our analyses suggest somewhat greater support for a sister relationship between *Lophonetta* and *Amazonetta-Speculanas*, but this conclusion requires additional testing.

Based on morphology, Livezey (1991) placed *Amazonetta* as the sister genus to *Callonetta*, and basal in the subtribe Anateae. In the same study, *Lophonetta* and *Speculanas* were inferred as sister species, comprising a clade sister to the "true" dabbling ducks (Livezey 1991). While *Speculanas* and *Lophonetta* are members of the same South American clade, our analysis indicates that these partially sympatric species are not sister taxa. The speculum is similar in both species, being bronze-colored with a posterior black and terminal white border, and lacking any anterior border differentiation (Johnsgard 1965). Johnsgard (1978) also found strong similarities in the displays of *Lophonetta* and *Speculanas*, as well as similarities in their tracheal structure. Both mitochondrial and nuclear sequence data, however, indicate that *Speculanas* is more closely related to *Amazonetta*, which is substantially smaller in size. This and other morphological differences might have arisen as these species diverged in distinct habitats in distant parts of South America. The larger bodied *Speculanas* inhabits the southern

Andes, whereas the smaller *Amazonetta* inhabits tropical regions, including the Amazon basin (see Fig. 1.1).

The placement of *Tachyeres* with these three genera by Johnson and Sorenson (1999) was surprising given the substantial morphological differences among them; morphologically, steamer ducks had been grouped with the shelducks (Livezey 1986a). Based on genetic data, however, it appears that morphology in steamer ducks is highly derived and divergent from other dabbling ducks. These large-bodied ducks likely evolved sympatrically in Patagonia with *Lophonetta* and *Speculanas*, although present day distribution patterns may not reflect their ancestral distribution.

Within *Tachyeres*, mtDNA suggests that *T. leucocephalus* and *T. patachonicus* are more closely related to each other than to *T. brachypterus*. These results conflict with phylogenies based on morphology (Livezey 1986b) and electrophoretic data (Corbin et al. 1988), which placed *T. patachonicus* as the sister group of all three flightless species, suggesting a single loss of flight. Our mtDNA data suggest that either (1) *Tachyeres* lost the capability of flight twice, or (2) flight was lost in ancestral *Tachyeres* and subsequently regained by *T. patachonicus*. MtDNA is just one locus, however, and nuclear loci provided little resolution of these relationships. Furthermore, the ability to fly in steamer ducks is dependent on a number of anatomical, behavioral, and environmental conditions (Livezey and Humphrey 1992). For example, Humphrey and Livezey (1982) found that up to 25% of male *T. patachonicus* from marine localities are permanently flightless. Flightlessness in steamer ducks probably evolved because of the year round

habitability of the southern South American marine littoral, making migration unnecessary (Livezey and Humphrey 1986).

The mtDNA data suggest a recent divergence of *T. leucocephalus* and *T. patachonicus*. After diverging from the two southernmost species (*T. pteneres*, *T. brachypterus*), the ancestral *T. patachonicus* population may have given rise to *T. leucocephalus* through a founder event at the periphery of its coastal range north of the Gulf of San Jorge in Chubut. The possibility that flightlessness evolved three times independently from a flying ancestor also should be considered. Large bodied *T. patachonicus* can be found year-round in marine habitats (Wilson et al. 2007), and some larger individuals are effectively flightless (Humphrey and Livezey 1982). Thus, a single, broadly distributed and flighted ancestor may have given rise to flightless descendents in three disjunct geographic areas (Fig. 1.1; but see Livezey 1986b). While perhaps less parsimonious in terms of morphological evolution, this hypothesis provides a simple explanation for current distributions.

In addition to incomplete lineage sorting, interspecific hybridization also might contribute to the lack of mtDNA monophyly among *Tachyeres* species (e.g., Peters et al. 2007). Waterfowl are well known for their capacity to hybridize and produce fertile offspring (Johnsgard 1960, Tubaro and Lijtmaer 2002). Indeed, it can be difficult to definitively identify steamer ducks using field marks or external characters in regions where they co-occur. Furthermore, there is evidence that maritime populations of *T. patachonicus* show "hybrid" characteristics of high genetic heterozygosity (Corbin 1983) and increased morphometric variability (Livezey 1986c). This highlights the importance

of sampling multiple individuals for species-level phylogenetics (Peters et al. 2005). Further studies with population-level sampling of each of the four *Tachyeres* species are needed.

In conclusion, relationships within this distinctive clade of South American ducks were not well resolved despite sequencing more than 10000 characters from six independent linkage groups. This lack of resolution likely resulted from high levels of homoplasy and a lack of informative characters (i.e., soft polytomies), rapid divergence times among genera and species (i.e., hard polytomies), or a combination of these factors. In the case of soft polytomies, it may be possible to resolve relationships by sequencing additional loci and applying phylogenetic methods that incorporate random lineage sorting and mutation (e.g., Edwards et al. 2007). In either case, it is clear that this group underwent at least two periods of rapid diversification, one producing the four genera and a more recent radiation among species of *Tachyeres*. This clade provides a striking example of closely related taxa that have radiated into morphologically and behaviorally divergent forms.

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Figure 1. 1 Geographic ranges of crested duck, Brazilian teal, spectacled duck, and the four species of steamer ducks. The range for flying steamer duck includes the coastal and inland habitats delimited by the bold line.

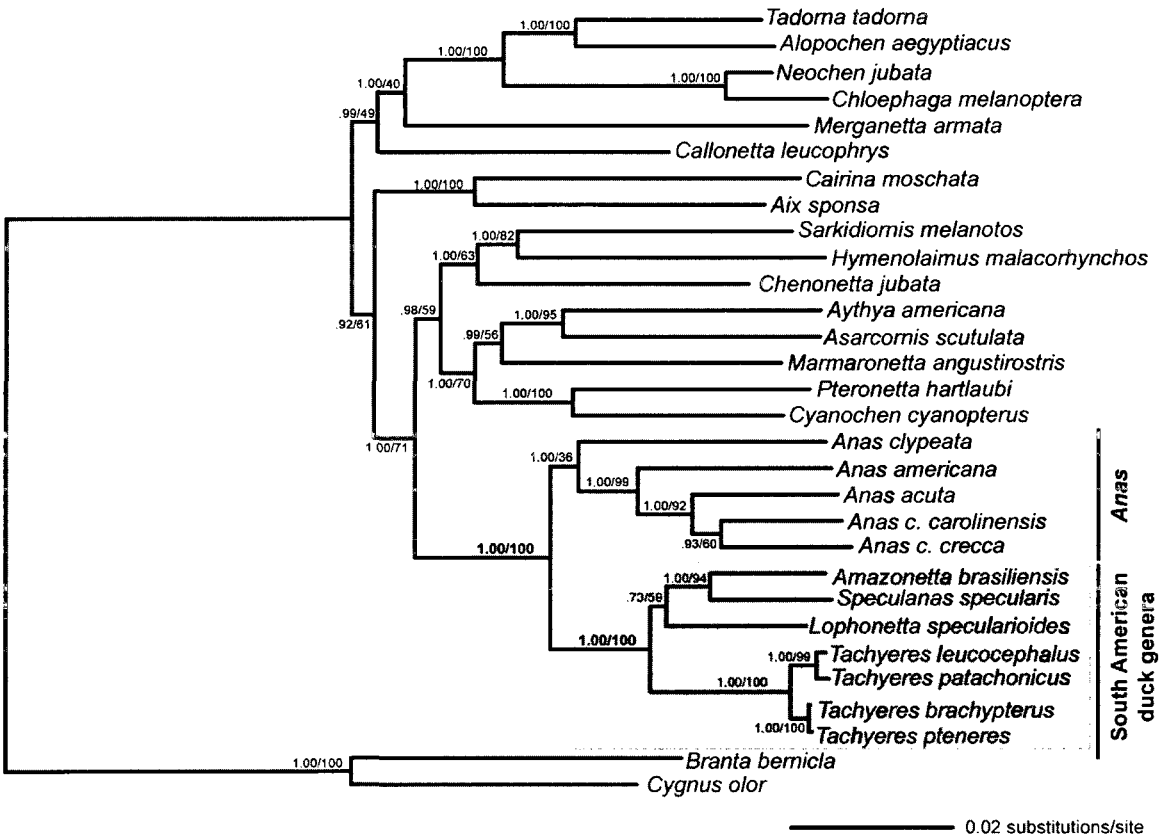

Figure 1.2 Bayesian 50% majority-rule tree showing the monophyly of *Amazonetta*, *Speculanas*, *Lophonetta*, and *Tachyeres* in relation to *Anas* and 18 other duck genera based on concatenated analysis of three mtDNA gene regions and five nuclear loci. Support for clades is indicated by the posterior probabilities/maximum parsimony bootstrap values. The best-fit model was GTR+I+G with I = 0.59 and $\alpha = 0.52$.

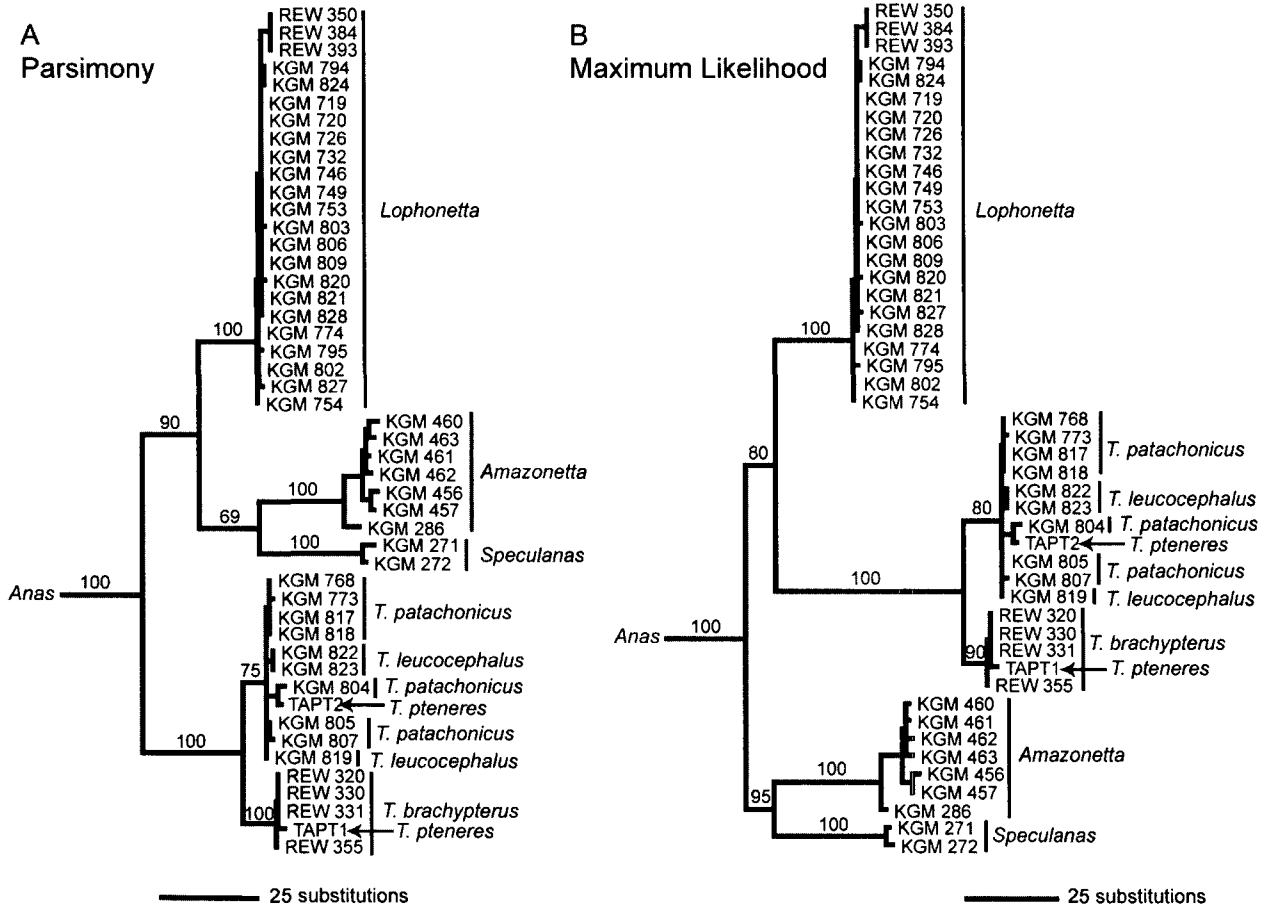

Figure 1.3 (A) One of 36 equally parsimonious trees derived based on 1314 characters from the mtDNA control region, tRNA-Phe, and 12S rRNA (length = 489, CI = 0.68). (B) One of four most likely trees derived for the same data set (–lnL = 3905.07). In both analyses, multiple trees differed only in intraspecific relationships. The best-fit model was TVM+I+G with I = 0.73 and α = 1.47. Bootstrap values above branches indicate support for clades.

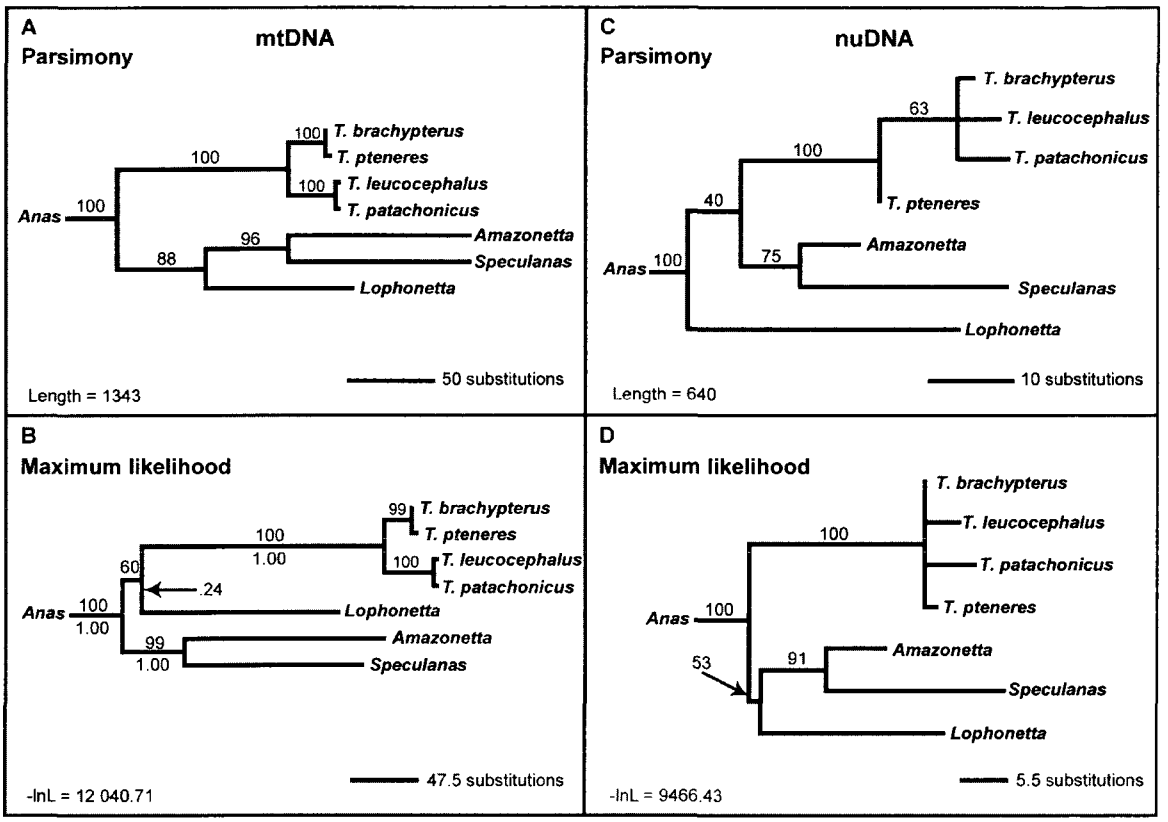

Figure 1.4 (A) Top left: Most parsimonious tree derived from 4468 characters of the combined data set from four mtDNA gene regions (tree length = 1343). (B) Bottom left: Maximum likelihood tree obtained for the same combined mitochondrial data set (–lnL = 12 040.71). The best-fit model for the combined mitochondrial data was GTR+I+G. (C) Top right: One of two parsimony trees (length = 640) for the concatenated six nuclear loci (the two trees differed in relationships among the four *Tachyeres* species). (D) Bottom right: Most likely tree (–lnL = 9466.43) for the nuclear data. The best-fit model for the concatenated nuclear loci was HKY+I with I = 0.89. Support values above and below branches correspond to nonparametric bootstrap, and Bayesian posterior probabilities, respectively.

Table 1.1 Number of positions, variable and informative positions, %GC, consistency index, rescaled consistency index, best-fit models, uncorrected percent of sequence divergence, and transition:transversion ratio for four mtDNA gene regions. Values reported are based on the one sequence per species dataset. The sequence divergence values exclude comparisons among *Tachyeres* species.

mtDNA locus	Positions	No. variable/ informative positions	%GC	CI	RC	Best fit model (AIC)	Uncorrected % sequence divergence	Ti/tv ratio[a]
Control region	1032	137/79	46.1	0.87	0.72	TVM + I + G	4.9–7.8	6.4
12S rRNA	1051	41/23	49.3	0.89	0.76	TVM + I	1.6–2.5	6.9
ND2	1095	127/54	50.1	0.93	0.80	TrN + I + G	4.7–6.9	15.6
ND5/cytochrome b	1290	147/75	50.0	0.86	0.66	TIM + G	5.2–7.5	21.7

[a]Estimates for the HKY85 model.

Table 1.2 Character state changes in the control region data supporting two alternative branching patterns for *Tachyeres* (TA), *Lophonetta* (LO), *Amazonetta* (AM), and *Speculanas* (SP).

Position	Substitution	Type	Steps	Consistency index
(TA (LO (AM, SP)))				
85	A → G	TI	4	0.25
113	T → C	TI	2	1.0
175	T → C	TI	4	0.5
176	C → T	TI	2	0.5
678	T → C	TI	1	1.0
762	G → A	TI	1	1.0
812	- → A	insertion	1	1.0
831	- → C	insertion	1	1.0
844	T → C	TI	2	0.5
1306	C → T	TI	1	1.0
Mean ± SD				0.78 ± 0.30
((TA, LO) (AM, SP))				
52	T → C	TI	3	0.33
259	C → A	TV	2	1.0
769	T → C	TI	1	1.0
777	A → G	TI	1	1.0
1252	G → A	TI	1	1.0
Mean ± SD				0.87 ± 0.30

Table 1.3 Log-likelihood for best-fit models selected using AIC and partitioned by codon position and locus for three possible resolutions of *Tachyeres* (TA), *Lophonetta* (LO), *Amazonetta* (AM), and *Speculanas* (SP).

	Best-fit model	(TA (LO (AM, SP)))	((TA, LO) (AM, SP))	(LO (TA (AM, SP)))	Parameters	AIC	*P*-values*
1st position	HKY + I	1541.02	**1540.04**	1541.02	5	3086.9	0.20
2nd position	HKY + I	**1118.05**	**1118.05**	**1118.05**	5	2234.14	0.98
3rd position	GTR + G	**3264.41**	3266.03	3266.03	9	6546.37	0.19
Control region (CR)	TVM + I + G	2418.41	**2415.72**	2418.41	9	6097.54	0.15
12S/tRNAs	TVM + I	**2141.80**	2141.96	2142.27	8	4297.68	0.43
ND2	TrN + I + G	**3142.97**	3143.09	3143.09	7	6295.84	0.33
ND5/cytb	TIM + G	**3552.04**	3552.30	3552.30	6	7113.87	0.27
Total mtDNA combined	GTR + I + G	**10092.88**	10092.89	10096.33	10	24104.44	0.34
Mixed 1 (CR, 12S, ND2, ND5/cytb)		11255.22	**11253.07**	11256.07	30	23804.93	0.29
Mixed 2 (1, 2, 3, CR, 12S)		10483.69	**10481.8**	10485.78	36	22262.63	0.17

*Minimum *P*-values for the approximately unbiased test of Shimodaira (2002).

Appendix 1.1 Locality and specimen information for South American ducks included in this study.

Museum catalog no.	Field catalog no.	Species	Date	Country	Province	Locality	Longitude	Latitude	Elevation (m)
UAM 17533	KGM 271	*Speculanas specularis*	19 Apr 01	Argentina	Neuquén	Río Chimehuin	–71.07170	–39.91610	1267
UAM 17532	KGM 272	*Speculanas specularis*	19 Apr 01	Argentina	Neuquén	Río Chimehuin	–71.07170	–39.91610	1267
UAM 14628	KGM 456	*Amazonetta brasiliensis*	15 Oct 01	Argentina	Salta	NE La Caldera	–65.37080	–24.55030	1468
UAM 14644	KGM 457	*Amazonetta brasiliensis*	15 Oct 01	Argentina	Salta	NE La Caldera	–65.37080	–24.55030	1468
UAM 14862	KGM 460	*Amazonetta brasiliensis*	15 Oct 01	Argentina	Corrientes	S Pedro Fernández	–58.99810	–28.72360	64
—	KGM 461	*Amazonetta brasiliensis*	18 Oct 01	Argentina	Corrientes	N Santa Lucia	–59.02690	–28.77250	45
UAM 14627	KGM 462	*Amazonetta brasiliensis*	18 Oct 01	Argentina	Corrientes	N Santa Lucia	–59.04780	–28.81170	45
UAM 14851	KGM 463	*Amazonetta brasiliensis*	18 Oct 01	Argentina	Corrientes	N Santa Lucia	–59.04780	–28.81170	45
UAM 17972	REW 320	*Tachyeres brachypterus*	15 Nov 02	Falkland Islands	East Falkland	Stanley Harbour	–57.86770	–51.69130	2
—	REW 330	*Tachyeres brachypterus*	23 Nov 02	Falkland Islands	East Falkland	Stanley Harbour	–57.86770	–51.69130	2
—	REW 331	*Tachyeres brachypterus*	23 Nov 02	Falkland Islands	East Falkland	Stanley Harbour	–57.86770	–51.69130	2
—	REW 355	*Tachyeres brachypterus*	16 Dec 02	Falkland Islands	East Falkland	Bertha's Beach, Fitzroy Farm	–58.38380	–51.89090	3
UAM 22621	KGM 768	*Tachyeres patachonicus*	29 Oct 03	Argentina	Santa Cruz	Estancia La Angostura	–70.69680	–48.62020	408
UAM 22625	KGM 773	*Tachyeres patachonicus*	30 Oct 03	Argentina	Santa Cruz	Laguna del Pescado	–72.92810	–49.12530	466
UAM 20715	KGM 804	*Tachyeres patachonicus*	7 Nov 03	Argentina	Santa Cruz	Puerto Santa Cruz	–68.50030	–50.06430	0

UAM 22624	KGM 805	*Tachyeres patachonicus*	7 Nov 03	Argentina	Santa Cruz
UAM 20714	KGM 807	*Tachyeres patachonicus*	9 Nov 03	Argentina	Santa Cruz
UAM 20799	KGM 817	*Tachyeres patachonicus*	11 Nov 03	Argentina	Chubut
UAM 22623	KGM 818	*Tachyeres patachonicus*	11 Nov 03	Argentina	Chubut
UAM 22622	KGM 819	*Tachyeres leucocephalus*	11 Nov 03	Argentina	Chubut
UAM 20801	KGM 822	*Tachyeres leucocephalus*	12 Nov 03	Argentina	Chubut
UAM 20800	KGM 823	*Tachyeres leucocephalus*	12 Nov 03	Argentina	Chubut
MDS	TAPT2	*Tachyeres pteneres*	1995	Chile	—
MDS	TAPT1	*Tachyeres pteneres*	—	Captive	—
UAM 19628	KGM 719	*Lophonetta specularioides*	20 Oct 03	Argentina	Chubut
UAM 19632	KGM 720	*Lophonetta specularioides*	20 Oct 03	Argentina	Chubut
UAM 19626	KGM 726	*Lophonetta specularioides*	22 Oct 03	Argentina	Chubut
UAM 19629	KGM 732	*Lophonetta specularioides*	23 Oct 03	Argentina	Chubut
UAM 22747	KGM 746	*Lophonetta specularioides*	26 Oct 03	Argentina	Santa Cruz
UAM 19636	KGM 749	*Lophonetta specularioides*	26 Oct 03	Argentina	Santa Cruz
UAM 20781	KGM 753	*Lophonetta specularioides*	28 Oct 03	Argentina	Santa Cruz
UAM 19627	KGM 754	*Lophonetta specularioides*	28 Oct 03	Argentina	Santa Cruz

Puerto Santa Cruz	–68.50030	–50.06430	0
Puerto Deseado	–65.88510	–47.75500	0
N Caleta Córdova	–67.36170	–45.72610	0
N Caleta Córdova	–67.36170	–45.72610	0
Bahía Bustamante	–66.53500	–45.13480	0
N Camarones	–65.69530	–44.75720	0
N Camarones	–65.69530	–44.75720	0
Navarino Island	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
RP 17, W Tecka	–71.06760	–43.60620	804
RN 40, S Tecka	–70.87550	–43.71010	934
RN 40, W Shaman	–70.67430	–44.38960	655
RN 40, N Río Mayo	–70.43980	–45.42210	578
RP 41, Estancia La Frontera	–71.86200	–46.84210	783
RN 40, ca Estancias Telken & La Paloma	–70.74550	–46.87610	618
RN 40, N Las Horquetas	–70.97490	–48.30230	540
RN 40, N Las Horquetas	–70.97490	–48.30230	540

UAM 19630	KGM 774	*Lophonetta specularioides*	31 Oct 03	Argentina	Santa Cruz
UAM 19625	KGM 794	*Lophonetta specularioides*	3 Nov 03	Argentina	Santa Cruz
UAM 19640	KGM 795	*Lophonetta specularioides*	5 Nov 03	Argentina	Santa Cruz
UAM 19631	KGM 802	*Lophonetta specularioides*	6 Nov 03	Argentina	Santa Cruz
UAM 19633	KGM 803	*Lophonetta specularioides*	6 Nov 03	Argentina	Santa Cruz
UAM 19635	KGM 806	*Lophonetta specularioides*	8 Nov 03	Argentina	Santa Cruz
UAM 19747	KGM 809	*Lophonetta specularioides*	10 Nov 03	Argentina	Chubut
UAM 19637	KGM 820	*Lophonetta specularioides*	11 Nov 03	Argentina	Chubut
UAM 19634	KGM 821	*Lophonetta specularioides*	11 Nov 03	Argentina	Chubut
UAM 19639	KGM 824	*Lophonetta specularioides*	12 Nov 03	Argentina	Chubut
UAM 19624	KGM 827	*Lophonetta specularioides*	13 Nov 03	Argentina	Chubut
UAM 19638	KGM 828	*Lophonetta specularioides*	13 Nov 03	Argentina	Chubut
—	REW 350	*Lophonetta specularioides*	9 Dec 02	Falkland Islands	East Falkland
—	REW 384	*Lophonetta specularioides*	28 Dec 02	Falkland Islands	East Falkland
—	REW 393	*Lophonetta specularioides*	31 Dec 02	Falkland Islands	East Falkland
MDS	—	*Anas crecca crecca*	—	Captive	—
MDS	—	*Anas crecca carolinensis*	1994	USA	California
MDS	—	*Anas americana*	1994	USA	Texas

Estancia Santa Margarita	–72.41400	–49.55810	246
RN 40, ca El Zurdo	–71.22580	–51.99600	122
RN 40, ca Estancia Monte Dinero	–68.66560	–52.26760	72
RN 3, ca Paraje Lemarchand	–69.48180	–50.75020	281
RP 288, ca Puerto Punta Quilla	–68.48800	–50.08890	3
Bahía Río Deseado	–65.97270	–47.74210	0
S Lago Colhué Huapí	–68.94000	–45.65240	267
Bahía Bustamante	–66.53500	–45.13480	0
Bahía Bustamante	–66.52120	–45.14930	0
S Camarones	–65.71630	–44.80330	0
Cabo Raso	–65.23010	–44.33410	0
Playa Bonita, S Rawson	–65.04820	–43.36090	0
Bertha's Beach, Fitzroy Farm	–58.38380	–51.89090	3
Bertha's Beach, Fitzroy Farm	–58.38380	–51.89090	3
Fitzroy, Fox Point	–58.38380	–51.89090	3
Sylvan Heights Waterfowl, NC	—	—	—
Solano County	—	—	—
Brazoria County	—	—	—

MDS	—	*Anas acuta*	—	Captive	—
UWBM 43948	—	*Anas acuta*	11 Jul 92	Russia	Chukotskiy Avtonomny y Okrug
MDS	—	*Anas clypeata*	1994	USA	Texas
UWBM 71262	—	*Anas clypeata*	26 May 94	Russia	Magadanska ya Oblast'
MDS	—	*Sarkidiornis melanotos*	—	Captive	—
MDS	—	*Cairina moschata*	—	Captive	—
MDS	—	*Aix sponsa*	—	Captive	—
MDS	—	*Tadorna tadorna*	—	Captive	—
MDS		*Chenonetta jubata*	—	Captive	—
MDS	—	*Callonetta leucophrys*	—	Captive	—
MDS LMTX R654	—	*Aythya americana*	—	United States	Texas
MDS	—	*Asarcornis scutulata*	—	Captive	—
MDS	—	*Pteronetta hartlaubi*	—	Captive	—
MDS	—	*Cyanochen cyanopterus*	—	Captive	—
MDS	—	*Marmaronetta angustirostris*	—	Captive	—
MDS	—	*Alopochen aegyptiacus*	—	Captive	—

National Zoological Park, DC	—	—	—
—	—	—	—
Henderson County	—	—	—
—	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Cedar Creek Natural History Area, MN	—	—	—
Laguna Madre	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—
Sylvan Heights Waterfowl, NC	—	—	—

MDS	—	*Neochen jubata*	—	Captive	—	Sylvan Heights Waterfowl, NC	—	—	—
MDS	—	*Chloephaga melanoptera*	—	Captive	—	Sylvan Heights Waterfowl, NC	—	—	—
UWBM 54417	—	*Merganetta armata*	5 Oct 1995	Argentina	Tucumán	Río Los Sosa, Route 307, km 20	—	—	650
MDS	—	*Hymenolaimus malacorhynchos*	—	New Zealand	—	Manganuiateao River	—	—	—
JFBM 38648	—	*Branta bernicla*	1990	United States	Alaska	Cape Pierce	—	—	—
MDS	—	*Cygnus olor*	—	Captive	—	Sylvan Heights Waterfowl, NC	—	—	—

Genbank accession numbers:
mtDNA control region HM063476–HM063528; 12S ribosomal RNA HM063529–HM063558; ND2 AF059114, AF059115, AF059116, AF059123, AF059124, AF059150, AF059157, AF059158, AF059159, AF059160, AF059161, AF059162, AF059163, AF059164, AF059170, AF059171, AF059172, AF059173, AF059174, AF090337, HM063559–HM063568; ND5/cytochrome *b* AF059053, AF059055, AF059062, AF059063, AF059064, AF059097, AF059098, AF059099, AF059100, AF059101, AF059103, AF059104, AF059110, AF059111, AF059112, AF059113, AF090337, HM063569–HM063573, HM063574–HM063581; LCAT HM063582–HM063611; CD4 HM063612–HM063640; PCK1-3 HM063641–HM063670; PCK1-9 HM063671–HM063699; HBA2 GQ271019, GQ271050, GQ271051, GQ271063, GQ271064, GQ271492, GQ271510, GQ271536, GQ271537, GQ271538, GQ271540, GQ271544, GQ271545, GQ271548, GQ271550, GQ271551, GQ271552, GQ271558, GQ271608, GQ271609, GQ271612, GQ271613, GQ271616, GQ271619, GQ271624, GQ271711, GQ271714, GQ271740, GQ271741; HBB GQ271807, GQ272272, GQ272273, GQ272276, GQ272277, GQ272280, GQ272283, GQ272310, GQ272312, GQ272318, GQ272319.

Appendix 1.2 Primers used to amplify and sequence mtDNA and nuclear DNA from the South American clade of ducks.

Locus	Primer	5′to 3′primer sequence	Reference
Control region	Duck.L81	TATTTGGYTATGYAYRTCGTGCAT	Muñoz-Fuentes et al. 2008
	H774	CCATATACGCCAACCGTCTC	Sorenson et al. 1999b
	L736	ATCTAAGCCTGGACACACCTG	Sorenson et al. 1999a
	H1530	GTGGCTGGCACARGATTTACC	
12S rRNA	L1263	YAAAGCATGRCACTGAA	Revised from Sorenson et al. 1999b
	H1859	TCGDTTRYAGRACAGGCTCCTCTA	Revised from Sorenson et al. 1999b
	L1754	TGGGATTAGATACCCCACTATG	Revised from Sorenson et al. 1999b
	H2294	TYTCAGGYGTARGCTGARTGCTT	Revised from Sorenson et al. 1999b
ND5/Cytochrome b	L14770	TAGGNCCNGARGGNYTNGC	
	H15295	CCTCAGAAKGATATYTGNCCTCAKGG	Sorenson et al. 1999b
	L14996	AAYATYTCWGYHTGATGAAAYTTYGG	McCracken & Sorenson 2005
	H15646	GGNGTRAAGTTTTCTGGGTCNCC	McCracken & Sorenson 2005
	L15413	GGGGGWTTYTCMGTNGAYAAYCC	McCracken & Sorenson 2005
	H16064	CTTCANTYTTTGGYTTACAAGRCC	Sorenson et al. 1999b
ND2	L5216	GGCCCATACCCCGRAAATG	McCracken & Sorenson 2005
	H5766	RGAKGAGAARGCYAGGATYTTKCG	McCracken & Sorenson 2005
	L5758	GGCTGAATRGGMCTNAAYCARAC	Sorenson et al. 1999b
	H6313	CTCTTATTTAAGGCTTTGAAGGC	Sorenson et al. 1999b
T-cell surface glycoprotein CD precursor (CD4)	CD4.4F	CTCCATCGATTAATNAGAACATCTCC	This study
	CD4.5R	TTCCKGAAGTTCAGAYGCCATGAC	This study

Lecithin cholesterol acyltransferase (LCAT) introns 2, 3, 4	LCAT2F	GTGGTGAACTGGATGTGCTACCG	This study
	LCAT3R	ACCTGCCAGTTTGCTCTGGTCCAG	This study
	LCAT3F	GTACCTGGCTTYGGCAAGACC	This study
	LCAT5Rb	CCCGATGTACTGATCTTTCCAGG	This study
Phosphoenolpyruvate carboxykinase (PCK1-3)	PCK1-3.F	GGTCGCTGGATGTCAGAAGAGG	McCracken & Sorenson 2005
	PCK1-3.RI	GYAGTAAAGGTGGGYGGAGG	
	PCK1-3.FI	GCAGCAGATAGCAARTGAGGTG	
	PCK1-3R	CCATGCTGAAGGGGATGACATAC	McCracken & Sorenson 2005
Phosphoenolpyruvate carboxykinase (PCK1-9)	PCK1-9.F	GGAGCAGCCATGAGATCTGAAGC	Sorenson et al. 2003
	PCK1-9.RI	CTTGAGAGCTGGCTTTCATTG	
	PCK1-9.FI	CTTACATTTTCTGTTCTGCTAGAGC	
	PCK1-9.R	GTGCCATGCTAAGCCAGTGGG	Sorenson et al. 2003
αA hemoglobin subunit (HBA2)	HBA2.14a.F	GGGCACCCGTGCTGGGGGCTGCCAAC	McCracken et al. 2009
	HBA2.373a.R	GCAGCCGCCACCTTCTTGCC	McCracken et al. 2009
	HBA2.342.F	GACCTACTTCCCCCACTTTGACC	McCracken et al. 2009
	HBA2.756.R	CTGGCAACAGGGTGGGTCCAGCTCTAGCC	McCracken et al. 2009
βA hemoglobin subunit (HBB)	HBB.1.F	GCCACACGCTACCCTCCACCCGACACC	McCracken et al. 2009
	HBB.646a.R	CCTGCCTSTCCTCSTGGTTCTKCC	McCracken et al. 2009
	HBB.482.F	GCTGCACGTGGACCCCGAGAACTTCAGG	McCracken et al. 2009
	HBB.1251a.R	TTTTTCTCCCTTCTGHCTTCATTTGG	McCracken et al. 2009
	HBB.1173.F	GCCAGTRGGAGCTTGCCCTTGGTGCC	McCracken et al. 2009
	HBB.1761.R	GGATGTTCTGGGAGCGTTGCTGCC	McCracken et al. 2009

Chapter 2:

Elevational variation in body size of Crested Ducks (*Lophonetta specularioides*) from the central high Andes, Mendoza, and Patagonia[1]

2.1 Abstract. – The Crested Duck (*Lophonetta specularioides*) inhabits the Andes of South America from Tierra del Fuego to central Perú, with two subspecies (*L. s. specularioides* and *L. s. alticola*) inhabiting different elevational environments in the Andes from sea level to 5,000 m. We evaluated morphological differences between the two subspecies of Crested Duck and evidence for Bergmann's and Allen's rules to gain a better understanding of the forces that have acted to shape geographic variation in morphology of highland and lowland populations. Overall body size of Crested Ducks differed between subspecies and between sexes. Male and female *L. s. alticola* from the central high Andes sampled at 3,338–4,611 m were larger than *L. s. specularioides* from southern Patagonia (< 934 m to sea level). *L. s. alticola* individuals of intermediate body size were found at mid elevations (1,522–2,552 m) in Mendoza, Argentina. Stepwise discriminant analysis (DA) classified 96.1% of *L. s. alticola* and 100% of *L. s. specularioides* males correctly; 100% of females were classified correctly. Body mass, wing chord, tarsus length, and bill length were positively correlated with elevation in

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[1] Bulgarella, M., R.E. Wilson, C. Kopuchian, T.H. Valqui, and K.G. McCracken. 2007. Elevational variation in body size of Crested Ducks (*Lophonetta specularioides*) from the central high Andes, Mendoza, and Patagonia. *Ornitología Neotropical* 18: 587–602.

male *L. s. alticola*, whereas total tarsus was negatively correlated with elevation in male *L. s. specularioides*. Crested Ducks conform to Bergmann's Rule. No evidence was found for Allen's Rule. Intermediate size Crested Ducks, such as those found in Mendoza, Argentina, might result from introgression between *L. s. alticola* and *L. s. specularioides*, and/or natural selection on body size of individuals locally adapted to intermediate elevational habitats.

2.2 INTRODUCTION

Similar morphological adaptations among species that live in similar environments are often expressed in terms of ecological or ecogeographic principles. Two of the most important ecogeographic principles are Bergmann's rule and Allen's rule (e.g., Mayr 1942). Bergmann's rule predicts that smaller-sized individuals are found in warmer parts of a species' range, and that larger individuals occur in cooler regions (Bergmann 1847). Allen's rule predicts that protruding body parts (e.g., tail, ears, bill) are relatively shorter in colder environments (Allen 1877). The usual explanation for Bergmann's and Allen's rules is that large animals with smaller extremities expend less energy for thermoregulation because of their smaller surface-to-volume ratio. Snow (1954a, 1954b) found widespread support for Bergmann's rule, but further expressed the principle as a "latitudinal effect", size being greater at higher than lower latitudes; and an "elevational effect", individuals living at higher elevations tend to be larger than those in lowlands. However, there are contrasting points of view regarding the physiological and ecological significance of these rules (Ray 1960, McNab 1971, James 1991). Bergmann's rule was critized by Scholander (1955), who argued that many species do not conform to it, and those examples that show clinal increases in body mass are physiologically not significant because vascular control and fur insulation are more efficient at heat dissipation and conservation than are changes in body size. Furthermore, some poikilotherms show similar body size trends with latitude and temperature (Ray 1960, Lindsey 1966), a pattern that may not easily be explained by heat conservation. James (1970) reformulated Bergmann's rule to account for the combined effects of several

climatic variables such as temperature and moisture, rather than temperature alone. Larger size in a hot dry climate would be optimum since heat loss by evaporation would be facilitated by the increased difference between the water vapor pressure of the evaporating surfaces and the vapor pressure of the inspired air. McNab (1971) argued that food availability also might explain why most homeotherms are larger at higher latitudes. He stated that mammals and birds living on mountains or islands are larger because there is low diversity of species in these regions, which may reflect an increase size of available food particles due to the reduced necessity of sharing a limited resource. More recently, Ashton (2002) found strong support for Bergmann's rule in birds, and Millien *et al.* (2006) concluded that patterns underlying ecotypic variation are complex, involve a number of interrelated variables, and are highly context-dependent.

Waterfowl inhabiting South America offer an excellent opportunity to evaluate evidence for Bergmann's and Allen's rules because they are distributed in the alpine wetlands and grasslands of the Andean Cordillera, which spans large elevational and latitudinal gradients. The Crested Duck (*Lophonetta specularioides*) is a partially migrant, sexually monomorphic, dabbling duck that is endemic to the central Andean and Patagonian regions of South America, and comprises two subspecies: Patagonian Crested Duck (*L. s. specularioides*) and Andean Crested Duck (*L. s. alticola*) (Phillips 1922–1926, Johnsgard 1978). The two subspecies inhabit different elevational environments ranging from 5000 meters in the central high Andes (*L. s. alticola*) to sea level in Patagonia and the Malvinas Islands (*L. s. specularioides*, Fig. 1). The subspecies are

reported to intergrade in an elevational transition zone at the latitude of Mendoza, Argentina, and Talca, Chile, respectively (Navas & Bo 1998).

Subspecies designations have been based on morphological and plumage differences. *L. s. specularioides* has red iris color, smaller body size, and more brownish or blackish mottled plumage, whereas *L. s. alticola* possesses yellow-orange iris color, larger body size, and more uniform washed out plumage with fewer breast spots (Phillips 1922–1926).

The objective of this study was to evaluate morphological differences between the two subspecies of Crested Duck and evaluate evidence for Bergmann's and Allen's rules to gain a better understanding of the forces that have acted to shape geographic variation in morphology of highland and lowland populations of Crested Ducks. We examined specimens collected throughout the geographic range of the species, with a much larger sample size and more extensive morphological measurements than previous studies.

2.3 METHODS

2.3.1 Specimen collecting and measurement. We collected 67 Crested Ducks (40 males and 27 females) from Andean regions of Argentina (2003, 2005), Bolivia (2005) and Peru (2002, 2006) (Appendix 2.1, Fig. 2.1). Specimens are archived at the University of Alaska Museum (Fairbanks, Alaska), Colección Boliviana de Fauna (La Paz, Bolivia), and Museo de Historia Natural de la Universidad de San Marcos (Lima, Perú).

Ten morphological measurements (± 0.1 mm, unless otherwise specified) were taken from each bird: wing chord length (WC, carpal joint to longest primary feather unflattened), tail length (TL), total tarsus length (TS1, top of bent knee to bottom of foot),

tarsus bone length (TS2), bill length (BL1, exposed culmen), bill length at nares (BL2), bill width at nares (BW), bill height (BH, height of upper mandible at posterior edge of nares), skull length (SK, back of the skull to tip of bill), and body mass (BM, ± 50 g). All measurements were taken the day the specimens were collected and before they were prepared. Sex was determined by dissecting the gonads.

Specimens were classified as either *L. s. specularioides* or *L. s. alticola* based on previously published plumage differences (Johnsgard 1978, Young 2005). All individuals collected from the highlands of Catamarca, Argentina, north to Peru were classified as *L. s. alticola*, and all individuals collected from Patagonia (coastal and inland) were classified as *L. s. specularioides*. All but one individual female (KGM 1221) from Mendoza, Argentina, were classified as *L. s. alticola* because these specimens had more uniform washed out plumage with few or no breast spots. Iris color faded too rapidly after collection to be consistently useful for identification.

2.3.2 Statistical analyses. Statistical analyses were performed on untransformed measurements using Statistica 6.0 (StatSoft 1995). Normality and homogeneity of variances were tested prior to the analysis. Multivariate analysis of variance (MANOVA) was performed to evaluate overall differences between subspecies and each sex. Following a significant MANOVA, we used analysis of variance (ANOVA) to test whether individual measurements differed between subspecies; significance levels were corrected for multiple comparisons using Bonferroni methods. We also performed principal components analysis (PCA) of the same ten measurements. The first three principal components (PC1–3) possessed eigenvalues greater than one (Kaiser 1960) and

were plotted separately for females and males. We used stepwise discriminant function analysis (DA) to determine the accuracy of subspecies identification (Sokal & Rohlf 1969, Sneath & Sokal 1973). DA was conducted separately for females and males. The final discriminant function included five measurements for males (WC, BL1, TS1, BW, BM) and six measurements (WC, BL1, BW, BH, SK, BM) for females.

Finally, we used partial correlation analysis of elevation and latitude with ten morphological measurements and PC1–3 to examine the joint relationship between elevation and latitude and morphological measurements, and to evaluate the partial correlation coefficients of each measurement and environmental variable (Sokal 1965). Analyses were performed separately for males and females within each subspecies. Significance levels were corrected for multiple comparisons using Bonferroni methods.

2.4 RESULTS

Overall body size of Crested Ducks differed between subspecies (Wilks' $\lambda = 0.25$, $F_{10, 54} = 15.5$, $P < 0.001$) and between sexes (Wilks' $\lambda = 0.51$, $F_{10, 54} = 5.1$, $P < 0.001$, Table 2.1). No significant interaction between subspecies and sex was observed ($P > 0.90$). Male and female *L. s. alticola* were significantly larger than male and female *L. s. specularioides*, respectively, for WC, TL, TS2, BL1, BL2, and SK (Table 2.1).

Three principal components with eigenvalues > 1 were retained in the principal components analysis and accounted for 78.4% of the total variance observed between subspecies in males and 81.1% in females, respectively. PC1 represented an overall difference in body size (male eigenvalue = 4.83, female eigenvalue = 5.00) and accounted for 48.3% and 50.0% of the variance for males and females, respectively. PC2

accounted for 16.5% of the variance in males (eigenvalue = 1.65) and corresponded to variations in TS1 and BH, whereas it accounted for 20.5% of the variance in females (eigenvalue = 2.05), and represented bill shape because BH and BW were the most highly correlated variables. PC3 (male eigenvalue = 1.34, female eigenvalue = 1.04) accounted for 13.5% and 10.4% of the variance for males and females, respectively, and represented a bill shape and body size difference between subspecies, as BW and BM were the most highly correlated variables in males. In females, TS1 and BM were the most influential variables (Table 2.2, Fig. 2.2). Plots of PC1 versus PC2 grouped all male *L. s. specularioides* and one *L. s. alticola* specimen (REW 130) together. It is possible that this individual is a subadult, as its measurements are smaller than the rest of the *L. s. alticola* specimens we sampled. Male *L. s. alticola* were more loosely scattered than *L. s. specularioides* in this plot, with specimens from Mendoza showing intermediate PC1 values. Plots of PC1 versus PC2 for females grouped one *L. s. specularioides* (KGM 1221) together with *L. s. alticola*; this female was from Mendoza (Fig. 2.2). Plots of PC1 versus PC3 showed similar differences between subspecies for both males and females.

Stepwise DA classified 96.1% of *L. s. alticola* and 100% of *L. s. specularioides* males correctly. The only male that was misclassified was a small body-sized Andean Crested Duck (REW 130; see above) from Perú that grouped with Patagonian Crested Ducks. Female *L. s. alticola* and *L. s. specularioides* were correctly classified 100% of the time. Clear separation of subspecies within each sex resulted from larger measures of WC, BL2, BW, and BM in *L. s. alticola.* BH and SK also contributed to differences between subspecies in females, as did TS1 in males.

Partial correlations coefficients for elevation and latitude regressed on all measurements are shown in Table 2.3. Most of the morphological measurements show a positive association with elevation in male *L. s. alticola*. In contrast, TS1 was negatively correlated with elevation for male and female *L. s. specularioides*, whereas in both sexes of *L. s. alticola,* TS1 was positively correlated with elevation ($P < 0.05$). In female *L. s. alticola*, PC3 was strongly correlated with elevation. After adjusting the significance level with Bonferroni corrections, only WC, TS1, TS2, PC1 and PC2 were positively correlated with elevation in male *L. s. alticola* ($P < 0.002$) (Table 2.3, Fig. 2.3).

L. s. alticola individuals collected from intermediate elevations in Mendoza (1,522–2,552 m) exhibited smaller body size than individuals collected from the Altiplano for all variables and showed strongly positive correlations with elevation (Fig. 2.3). For example, the r^2 values for TS1 and TS2 vs elevation were > 0.90 in male *L. s. alticola* from Mendoza, with an increase in TS1 from 48 mm at 1,522 m to 60 mm at 2,552 m, an overall increase in elevation of only ~1000 m (Fig. 2.3). *L. s. alticola* males inhabiting higher elevations in the Altiplano (> 3,500 m) showed a significant increase of WC with elevation ($r^2 = 0.22$, $P < 0.05$) in an elevational gradient of 700 m, from an altitude of 3,900 m to 4,600 m (Fig. 2.3).

No measurements showed significant correlations with latitude for females (Table 2.3). For males, PC2 was negatively correlated with latitude in *L. s. alticola*, and TS2 was positively correlated with latitude for *L. s. specularioides*, but after Bonferroni correction, neither was significant.

2.5 DISCUSSION

Explanations for the evolution of geographic variation within species state that populations inhabiting different localities are subject to different ecological and climatic pressures, thus giving rise to phenotypic distinctions (e.g., Mayr 1963). Phenotypic differences are maintained in part by the reduction of gene flow among populations separated by large distances and/or physical-ecological barriers (Gould & Johnston 1972). Empirical support relating morphometric variation and regional climates in birds is provided by a number of studies (e.g., Rand 1936, James 1970, Power 1970, Niles 1973). On the other hand, Remsen (1984) pointed out the influence of random processes in the differentiation of polytypic bird species in the Andes; the appearance of phenotypic changes, at different times and rates, at random with respect to geography.

It has long been recognized that an increase in body size frequently correlates with an increase in latitude and elevation (Bergmann 1847, Rand 1936, Traylor 1950, Snow 1954b). This trend is most often explained by thermoregulatory advantages of being larger in colder environments; larger animals face smaller heat losses in cold climates because of their proportionally smaller surface areas (Randall *et al.* 2002). Millien *et al.* (2006), however, argued that the patterns underlying geographic variation are complex and highly context-dependent, reducing the 'predictive-power' of ecogeographical rules. Geographic variation in size of Crested Ducks can be explained as a combination of processes leading to the observed overall trend of larger bodied individuals at higher elevations in the Andes (3,000–5.000 m), with the coastal and inland specimens from

Patagonia being smaller, and Mendoza specimens being intermediate in size between the two populations.

Patagonia and the central high Andes have similar cold, semi-arid climates, associated with strong winds year-round, with elevation and air density being the main differences. Northern Patagonia is semiarid, with minimum annual mean temperatures from –11 to –5°C, and prevailing cold, dry and strong southwest winds. Southern Patagonia becomes increasingly peninsular with higher latitude, with minimum annual temperatures between –9 and –33°C. The climate is cold and dry, with strong west winds. Frosts can occur throughout the year; spring and autumn provide only short transitions between summer and winter (Encyclopedia Britannica 2006). The Altiplano climate is semiarid, with reported mean minimum temperatures of 5°C at Cusco, Perú (3,248 m), 1°C at La Paz, Bolivia (4,012 m) and –8°C at La Quiaca, Argentina (3,461 m; Weatherbase 2007).

In our study, the low air density and oxygen supply (~60% of sea level at 4000 m) associated with high elevation, may be part of the explanation of why the Andean subspecies is larger. Aldrich & James (1991) and James (1991) described a negative relationship between size in birds and either wet-bulb temperature, vapor pressure, or absolute humidity (all measurements sensitive to both temperature and moisture). Their model, thus, accounts for the fact that size tends to increase in arid regions, independently of latitude and altitude, and that widespread species tend to be largest in areas that are high, cool, and dry, such as the high Andes. James' modification better supported Bergmann's rule in other studies including birds and mammals (Wigginton & Dobson 1999, Meiri & Dayan 2003). Snow (1954b) stated that wings of montane bird species are

longer not only because they have larger overall body size, but also because the thinner air at high altitudes necessitates a relatively larger wing for efficient flight. Moreover, Hopkins & Powell (2001) found a relationship between body size and P_{50} (the oxygen partial pressure at which hemoglobin is half saturated), with small animals generally having lower affinity hemoglobin than larger animals. Smaller animals have a higher mass specific metabolic rate and may be selected to favor oxygen unloading to the tissues (Hopkins & Powell 2001). Therefore, even when there are no thermal gradients among populations, Crested Ducks conform to Bergmann's rule.

According to Allen's rule, heat will be conserved most efficiently in colder climates if protruding parts, from which heat loss is most rapid, are reduced (Snow 1954b). *L. s. specularioides* and *L. s. alticola* showed conflicting relationships between tarsus length and elevation. Specifically, male and female *L. s. alticola* increased TS1 with increasing elevation, whereas both sexes of *L. s. specularioides* showed decreased TS1 with increasing elevation. Bill length also increased with elevation for male *L. s. alticola*. No other consistent pattern of appendage variation with elevation or latitude was observed; therefore, Crested Ducks do not seem to conform to Allen's rule.

Little is known about the elevational movements of the different populations of Crested Ducks. This information is vital when analyzing trends within and between subspecies. Patagonian Crested Ducks are mostly sedentary, even in the southern parts of their range. Farther north Andean Crested Ducks from the Bolivian and Peruvian Andean lakes breed in the Cordilleras and descend in winter (Delacour 1954); they may migrate as low as to 2,000 m (Young 2005). Evidence also shows that Crested Ducks are encountered in both

extremes of the range in the very same season (Phillips 1922–1926). Little snow accumulates in the central high Andean plateau, so many Crested Ducks remain high in the Altiplano year-round. In contrast, the Andes in Mendoza are narrower and snow-covered much of the year, however, no information is available about how regional variation in vertical movement influences the biology and morphology of this species.

Morphologically intermediate populations of Crested Ducks, such as those found in Mendoza, Argentina, might be interpreted as evidence for introgression between the two populations, i.e., Andean Crested Ducks in the north and Patagonian Crested Duck populations in the south (Navas & Bo 1998). Intermediate morphology might also be maintained by natural selection on body size of individuals locally adapted to inhabiting intermediate or a range of different elevational environments.

Finally, did Crested Ducks diverge from other duck species in the lowlands and then colonize the highlands spreading north through Mendoza, thus increasing in body size as they adapted to a new highland environment? Or did Crested Ducks originate in the central high Andes and disperse south and to the lowlands? Additional genetic and physiological analyses will be necessary to determine historical directions of colonization of the Andes and identify factors related to body size that may be of selective advantage to Crested Ducks inhabiting different elevational environments.

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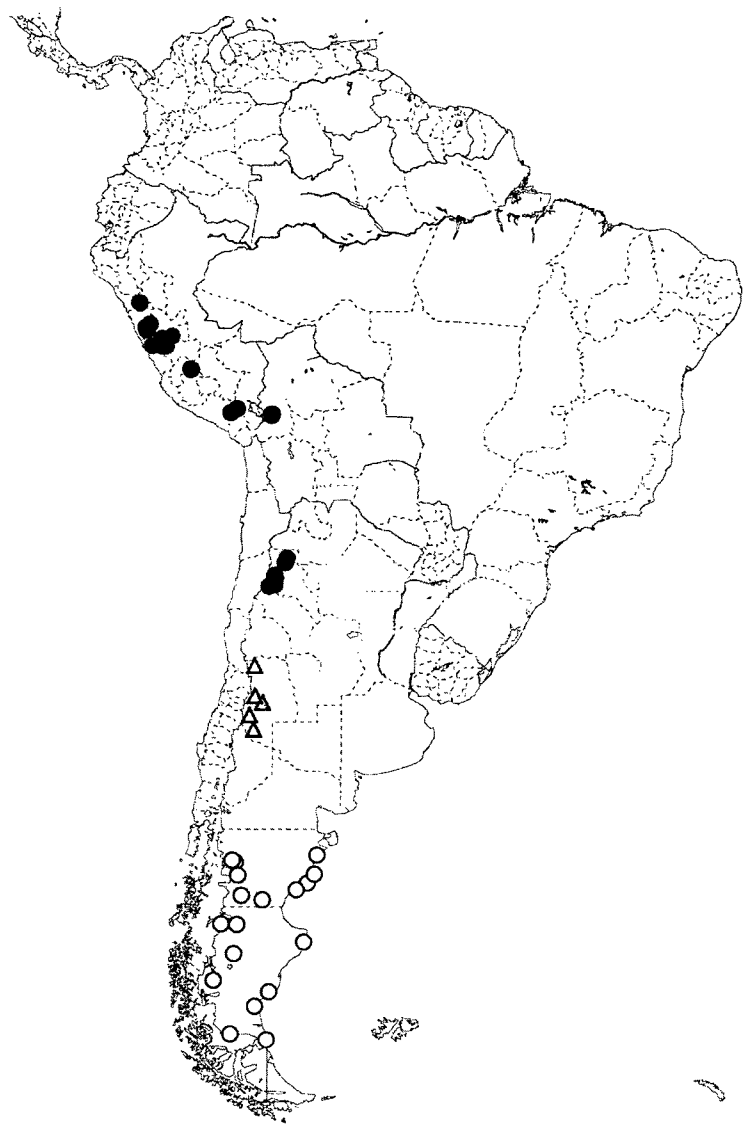

Figure 2.1 Geographic distribution of Crested Duck specimens collected between 2002 and 2006. Black circles represent the highland subspecies *L. s. alticola* from the central high Andes, triangles represent *L. s. alticola* from Mendoza, and white circles represent the lowland subspecies *L. s. specularioides*.

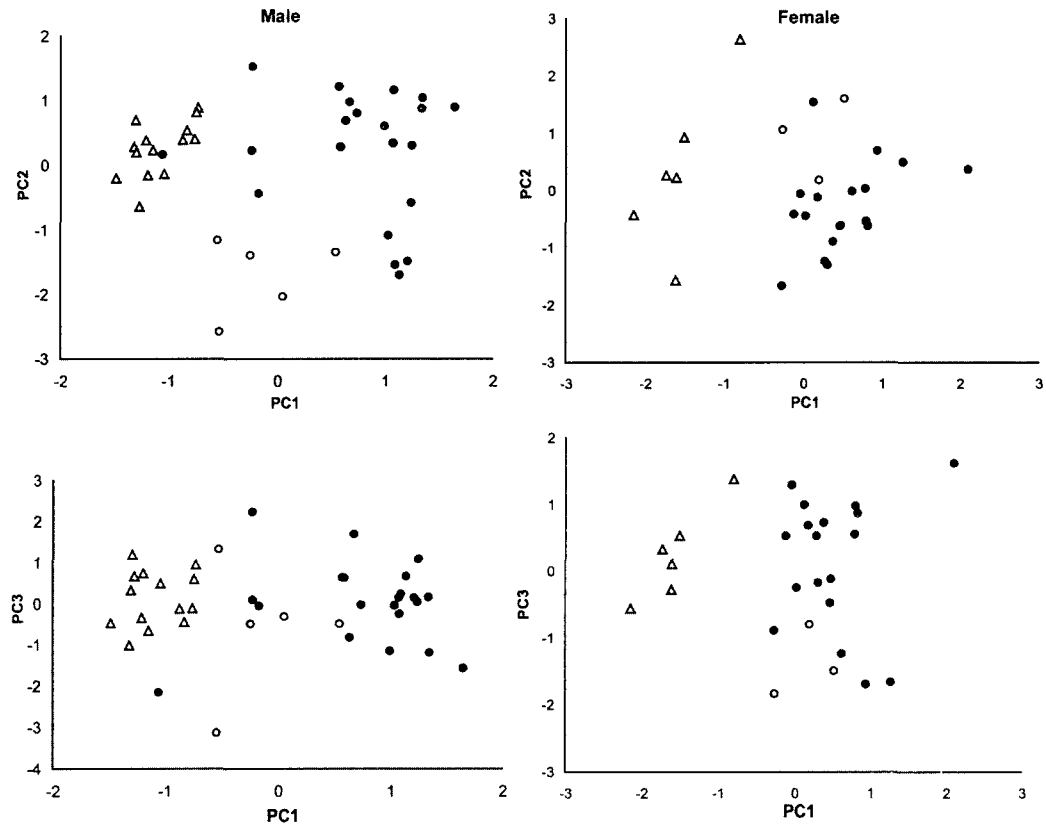

Figure 2.2 Principal components (PC1 vs PC2 and PC1 vs PC3) analysis of 10 body size measurements for female and male Crested Ducks (*Lophonetta specularioides*). Black circles represent *L. s. alticola* from the central high Andes, white circles represent *L. s. alticola* from Mendoza, and triangles represent *L. s. specularioides* from Patagonia.

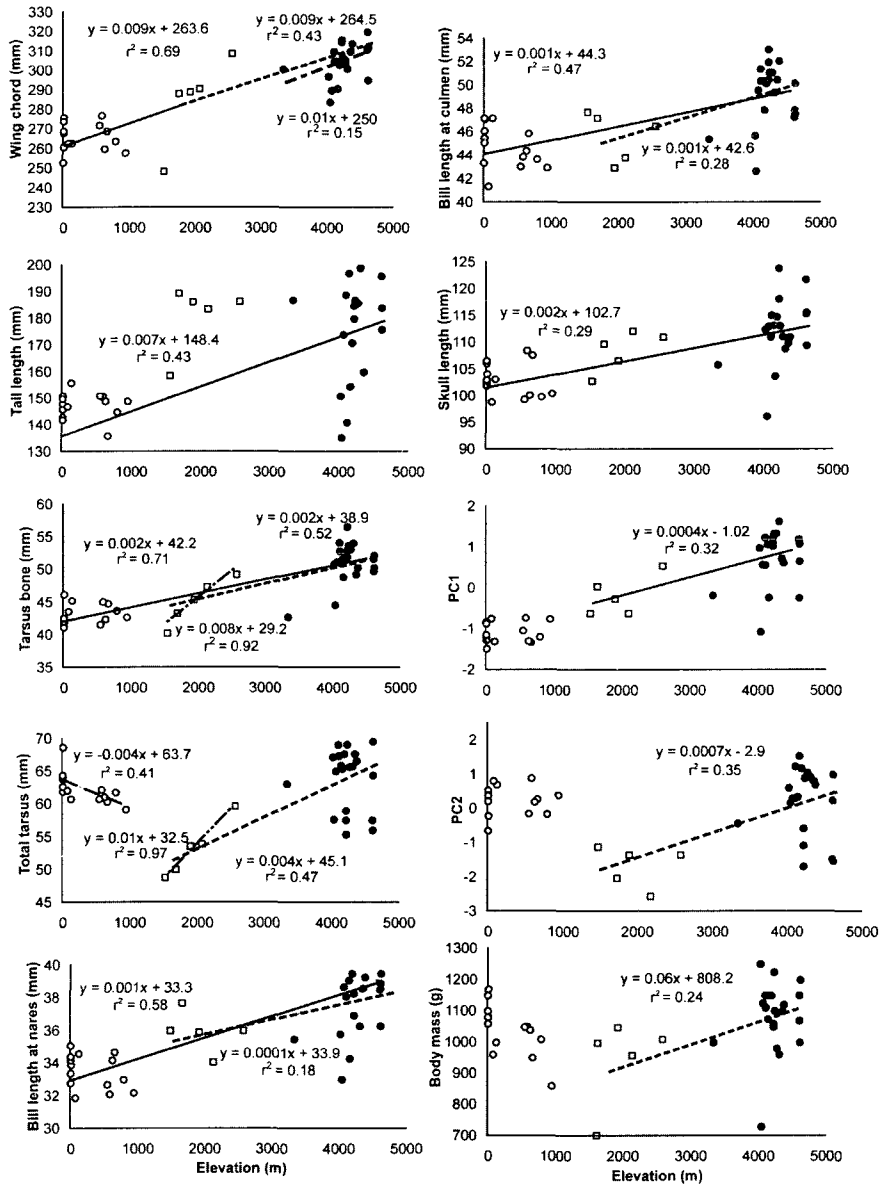

Figure 2.3 Linear regressions of body size measurements and elevation for male Crested Ducks. Black circles represent *L. s. alticola* from the central high Andes, white squares represent *L. s. alticola* from Mendoza, and white circles represent *L. s. specularioides* from Patagonia. The regression lines for *L. s. alticola* and *L. s. alticola* & *L. s. specularioides* (combined) are overlaid in the wing chord graph.

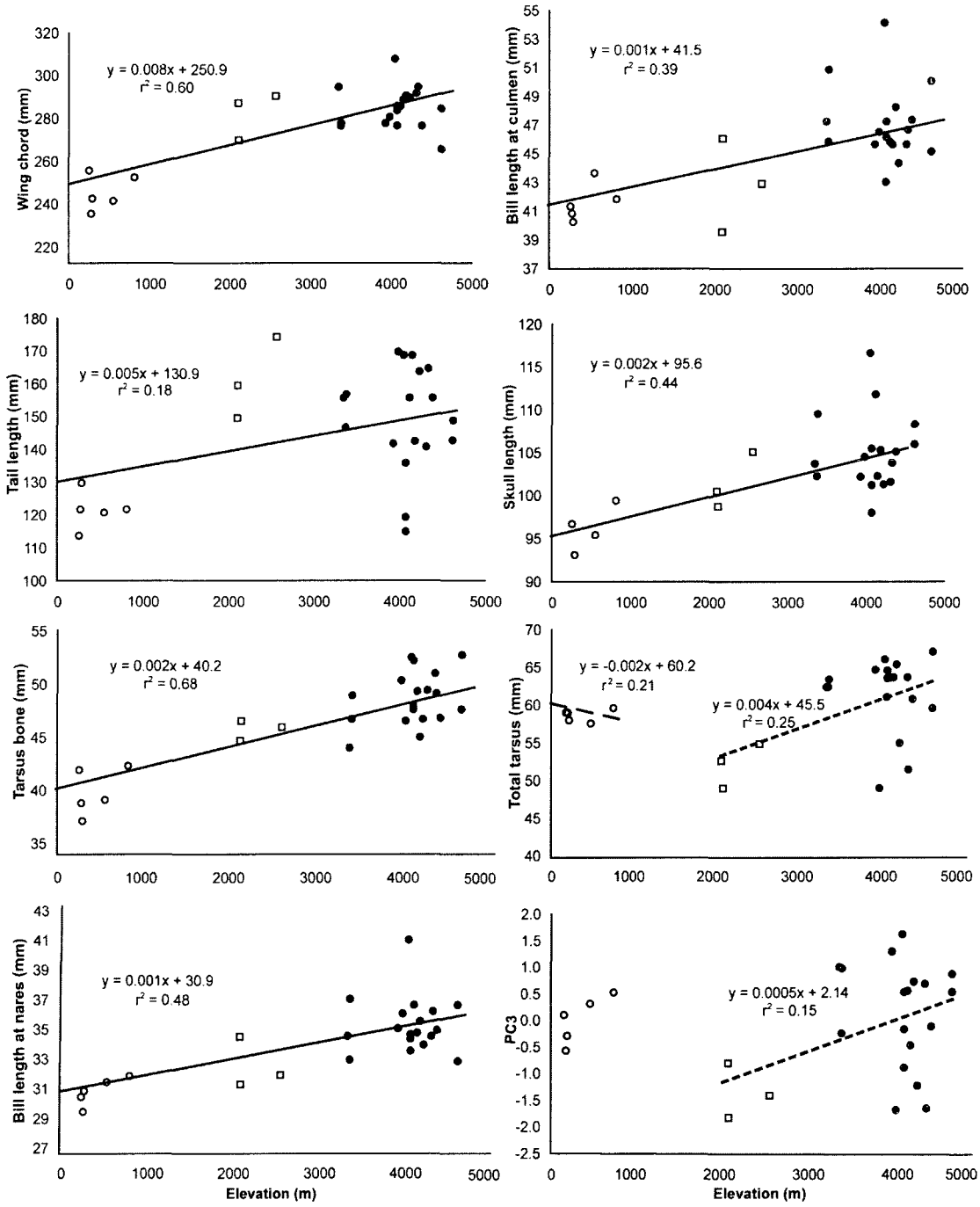

Figure 2.4 Linear regressions of body size measurements and elevation for female Crested Ducks. Black circles represent *L. s. alticola* from the central high Andes, white squares represent *L. s. alticola* from Mendoza, and white circles represent *L. s. specularioides* from Patagonia.

Table 2.1 Measurements (mm) and body mass (g) of Crested Duck subspecies.

Male		***L. s. alticola*** **(n = 26)**			***L. s. specularioides*** **(n = 14)**		
	P*	**Mean**	**SE**	**Range**	**Mean**	**SE**	**Range**
Body mass (BM)	0.66	1056.5	25.0	1004.9–1108.1	1040.0	21.7	993.1–1086.9
Wing cord (WC)	0.001	300.9	2.7	295.2–306.6	266.2	1.9	262.1–270.3
Tail (TL)	0.001	179.6	3.7	171.9–187.4	147.4	1.3	144.6–150.2
Total tarsus (TS1)	0.76	61.9	1.2	59.4–64.4	62.4	0.6	61.1–63.7
Tarsus bone (TS2)	0.001	50.0	0.7	48.4–51.6	43.2	0.4	42.3–44.1
Bill length at nares	0.001	37.7	0.4	36.8–38.6	33.5	0.3	32.8–34.1
Bill length at culmen	0.001	48.6	0.5	47.5–49.8	44.8	0.5	43.7–45.8
Bill height (BH)	0.09	17.2	0.3	16.5–17.9	16.3	0.2	15.8–16.9
Bill width at nares (BW)	0.98	19.6	0.2	19.1–20.0	19.5	0.2	19.2–19.9
Skull length (SK)	0.001	110.6	1.4	107.7–113.5	103.1	0.8	101.2–104.9
Female		**(n = 20)**			**(n = 7)**		
Body mass	0.42	966.0	24.7	914.2–1017.7	926.4	42.9	821.4–1031.4
Wing cord	0.001	285.6	2.0	281.3–289.8	252.5	4.9	240.4–264.7
Tail	0.005	151.6	3.6	144.0–159.2	130.1	5.6	116.2–144.0
Total tarsus	0.31	60.6	1.3	58.0–63.4	58.4	1.1	55.7–60.9
Tarsus bone	0.001	48.3	0.6	47.1–49.5	41.5	1.2	38.5–44.5
Bill length at nares	0.001	34.9	0.5	34.0–36.0	31.8	0.6	30.1–33.4
Bill length culmen	0.006	46.4	0.7	45.0–47.9	42.7	0.8	40.7–44.7
Bill height	0.78	15.5	0.3	14.9–16.2	15.7	0.6	14.3–17.1
Bill width at nares	0.32	18.3	0.1	18.1–18.6	18.7	0.4	17.6–19.8
Skull length	0.001	104.7	0.9	102.7–106.8	96.7	1.6	92.6–100.7

*ANOVA *P*-value for subspecies effect.

Table 2.2 Principal components (PC1–3) for 10 body size measurements of male and female Crested Ducks. Bold text indicates variables with a strong association ($|r| > 0.7$) with the principal component.

	Male			Female		
	PC1	PC2	PC3	PC1	PC2	PC3
Body mass	0.34	0.23	**0.73**	0.40	0.30	**0.72**
Wing chord	**0.90**	-0.05	0.09	**0.90**	0.11	0.00
Tail	**0.81**	-0.38	-0.05	**0.74**	0.42	-0.22
Total tarsus	0.21	**0.82**	0.38	0.12	-0.35	**0.82**
Tarsus bone	**0.89**	0.10	0.20	**0.83**	-0.13	0.21
Bill length at nares	**0.91**	0.00	0.09	**0.88**	-0.03	0.34
Bill length at culmen	**0.87**	0.15	0.09	**0.77**	-0.09	0.49
Bill height	0.26	**-0.77**	0.39	0.19	**0.79**	-0.22
Bill width	-0.02	-0.12	**0.88**	-0.13	**0.87**	0.11
Skull length	0.63	-0.03	0.28	**0.84**	0.11	0.37
Eigenvalue	4.83	1.65	1.34	5.00	2.05	1.04
% of variance	48.3	16.5	13.4	50.0	20.5	10.4
Cumulative %	48.3	64.9	78.4	50.0	70.6	81.1

Table 2.3 Partial correlation coefficients of body size measurements, PC1, PC2, and PC3 versus elevation and latitude for Crested Ducks. Significant correlations ($P < 0.05$) indicated in bold text. Correlations significant after Bonferroni correction are indicated by an asterisk.

	Male		Female	
Elevation	*L. s. alticola*	*L. s.*	*L. s. alticola*	*L. s.*
Body mass (BM)	**0.47**	-0.38	0.31	-0.12
Wing chord (WC)	**0.64***	-0.14	-0.29	-0.23
Tail (TL)	-0.01	0.10	-0.12	-0.41
Total tarsus (TS1)	**0.71***	**-0.56**	**0.56**	**-0.91**
Tarsus bone (TS2)	**0.71***	-0.50	0.34	-0.34
Bill length at nares (BL1)	**0.43**	-0.32	0.22	-0.07
Bill length at culmen	**0.53**	-0.38	0.28	-0.11
Bill height (BH)	-0.32	-0.20	-0.42	-0.65
Bill width (BW)	0.01	-0.30	-0.36	-0.73
Skull length (SK)	0.21	-0.43	0.23	-0.19
PC1	**0.58***	-0.41	-0.01	0.11
PC2	**0.67***	-0.22	-0.41	-0.71
PC3	0.23	-0.45	**0.50**	-0.61
Latitude				
Body mass	0.15	0.03	0.28	-0.18
Wing chord	0.32	0.12	-0.29	-0.52
Tail	0.35	-0.15	0.11	-0.64
Total tarsus	-0.28	0.31	0.42	-0.69
Tarsus bone	0.05	**0.58**	-0.07	-0.71
Bill length at nares	-0.11	0.20	-0.13	-0.51
Bill length at culmen	-0.02	0.23	-0.07	-0.55
Bill height	0.35	0.12	-0.23	-0.74
Bill width	0.09	0.22	0.14	-0.72
Skull length	0.17	0.40	0.12	-0.42
PC1	0.13	0.34	-0.25	-0.50
PC2	-0.43	0.08	0.04	-0.73
PC3	0.14	0.25	0.38	-0.43

Appendix 2.1 Locality and specimen information for Crested Ducks included in this study.

UAM no.	Field catalog no.	Date	Country	Province/ department	Locality	Longitude	Latitude	Elevation (m)	Subspecies	Sex
22741	REW-094	11 Aug 2002	Perú	Junín	S Junín, Carretera Central, km 218	-75.94180	-11.23270	4177	alticola	F
	REW-104	12 Aug 2002	Perú	Pasco	ca Cerro de Pasco	-76.89150	-11.20410	4234	alticola	M
20829	REW-107	12 Aug 2002	Perú	Pasco	ca Cerro de Pasco	-76.22320	-10.69380	4188	alticola	M
	REW-130	23 Aug 2002	Perú	Ancash	Laguna Conococha	-77.28350	-10.11970	4039	alticola	M
	REW-131	23 Aug 2002	Perú	Ancash	Laguna Conococha	-77.28350	-10.11970	4039	alticola	F
	REW-139	25 Aug 2002	Perú	Ancash	Laguna Tapara, SE Pachacoto	-77.36490	-9.90790	4065	alticola	F
	REW-140	25 Aug 2002	Perú	Ancash	Laguna Tapara, SE Pachacoto	-77.36490	-9.90790	4065	alticola	M
	REW-160	30 Aug 2002	Perú	Ancash	Laguna Canrash	-77.05580	-9.68520	4270	alticola	M
20793	REW-213	5 Oct 2002	Perú	Ayacucho	Razuhuilca, ca Huanta	-74.17310	-12.87980	4100	alticola	M
	REW-214	6 Oct 2002	Perú	Ayacucho	Razuhuilca	-74.15460	-12.91380	4065	alticola	F
	REW-215	6 Oct 2002	Perú	Ayacucho	Razuhuilca	-74.15460	-12.91380	4065	alticola	F
20784	REW-289	21 Oct 2002	Perú	Puno	Laguna Lagunillas	-70.81310	-15.69090	4157	alticola	M

20789	REW-290	23 Oct 2002	Perú	Arequipa	ca Imata
19628	KGM-719	20 Oct 2003	Argentina	Chubut	RP 17, W Tecka
19632	KGM-720	20 Oct 2003	Argentina	Chubut	RN 40, S Tecka
19626	KGM-726	22 Oct 2003	Argentina	Chubut	RN 40, W Shaman
19629	KGM-732	23 Oct 2003	Argentina	Chubut	RN 40, N Río Mayo
22747	KGM-746	26 Oct 2003	Argentina	Santa Cruz	RP 41, Estancia La Frontera
19636	KGM-749	26 Oct 2003	Argentina	Santa Cruz	RN 40, ca Estancias Telken & La Paloma
20781	KGM-753	28 Oct 2003	Argentina	Santa Cruz	RN 40, N Las Horquetas
19627	KGM-754	28 Oct 2003	Argentina	Santa Cruz	RN 40, N Las Horquetas
19630	KGM-774	31 Oct 2003	Argentina	Santa Cruz	Estancia Santa Margarita, ca Lago Viedma
19625	KGM-794	3 Nov 2003	Argentina	Santa Cruz	RN 40, ca El Zurdo
19640	KGM-795	5 Nov 2003	Argentina	Santa Cruz	RN 40, ca Estancia Monte Dinero
19631	KGM-802	6 Nov 2003	Argentina	Santa Cruz	RN 3, ca Paraje Lemarchand
19633	KGM-803	6 Nov 2003	Argentina	Santa Cruz	RP 288, ca Puerto Punta Quilla
19635	KGM-806	8 Nov 2003	Argentina	Santa Cruz	Bahía Río Deseado

-71.24380	-15.97340	4349	alticola	M
-71.06760	-43.60620	804	specularioides	F
-70.87550	-43.71010	934	specularioides	M
-70.67430	-44.38960	655	specularioides	M
-70.43980	-45.42210	578	specularioides	M
-71.86200	-46.84210	783	specularioides	M
-70.74550	-46.87610	618	specularioides	M
-70.97490	-48.30230	540	specularioides	F
-70.97490	-48.30230	540	specularioides	M
-72.41400	-49.55810	246	specularioides	F
-71.22580	-51.99600	122	specularioides	M
-68.66560	-52.26760	72	specularioides	M
-69.48180	-50.75020	281	specularioides	F
-68.48800	-50.08890	3	specularioides	M
-65.97270	-47.74210	0	specularioides	M

19747	KGM-809	10 Nov 2003	Argentina	Chubut	S Lago Colhué Huapí
19637	KGM-820	11 Nov 2003	Argentina	Chubut	Bahía Bustamante
19634	KGM-821	11 Nov 2003	Argentina	Chubut	Bahía Bustamante
19639	KGM-824	12 Nov 2003	Argentina	Chubut	S Camarones
19624	KGM-827	13 Nov 2003	Argentina	Chubut	Cabo Raso
19638	KGM-828	13 Nov 2003	Argentina	Chubut	Playa Bonita, S Rawson
22749	KGM-1073	4 Nov 2005	Argentina	Catamarca	Laguna Antofagasta, Antofagasta de la Sierra
22744	KGM-1074	4 Nov 2005	Argentina	Catamarca	Laguna Antofagasta, Antofagasta de la Sierra
22739	KGM-1087	7 Nov 2005	Argentina	Catamarca	Río Punilla, 35 km N Antofagasta de la Sierra
22738	KGM-1088	7 Nov 2005	Argentina	Catamarca	Río Punilla, 35 km N Antofagasta de la Sierra
22743	KGM-1122	12 Nov 2005	Argentina	Catamarca	Río Chaschuil, S La Gruta
22745	KGM-1139	13 Nov 2005	Argentina	Catamarca	Río Chaschuil, ca Embalse Cortaderas
22748	KGM-1140	14 Nov 2005	Argentina	Catamarca	Río Chaschuil, ca Embalse Cortaderas

-68.94000	-45.65240	267	specularioides	F
-66.53500	-45.13480	0	specularioides	F
-66.52120	-45.14930	0	specularioides	M
-65.71630	-44.80330	0	specularioides	M
-65.23010	-44.33410	0	specularioides	M
-65.04820	-43.36090	0	specularioides	M
-67.42409	-26.11280	3338	alticola	M
-67.42409	-26.11280	3338	alticola	F
-67.28391	-25.82775	4140	alticola	F
-67.28391	-25.82775	4140	alticola	M
-68.06677	-27.02894	3923	alticola	F
-68.14524	-27.56000	3363	alticola	F
-68.14498	-27.55590	3369	alticola	F

22735	KGM-1159	15 Nov 2005	Argentina	Catamarca	Laguna de los Aparejos
22751	KGM-1160	15 Nov 2005	Argentina	Catamarca	Laguna de los Aparejos
22737	KGM-1184	17 Nov 2005	Argentina	Catamarca	La Gruta
22742	KGM-1211	29 Nov 2005	Argentina	Mendoza	E Los Penitentes
22746	KGM-1212	29 Nov 2005	Argentina	Mendoza	E Los Penitentes
22734	KGM-1218	2 Dec 2005	Argentina	Mendoza	NW El Sosneado
22740	KGM-1220	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado
23413	KGM-1221	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado
22733	KGM-1224	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado
22736	KGM-1228	3 Dec 2005	Argentina	Mendoza	Pampa del Rodeo, 45 km SW Malargüe, RN 40
22750	KGM-1232	4 Dec 2005	Argentina	Mendoza	Río Grande
23412	REW-709	27 Nov 2005	Bolivia	La Paz	Laguna Khara Kkota
23415	REW-710	27 Nov 2005	Bolivia	La Paz	Laguna Khara Kkota
23411	REW-714	27 Nov 2005	Bolivia	La Paz	Laguna Kkota
23416	REW-715	27 Nov 2005	Bolivia	La Paz	Laguna Kkota

-68.54215	-27.64755	4106	alticola	M
-68.54215	-27.64755	4106	alticola	F
-68.14566	-26.92542	4020	alticola	M
-69.80995	-32.85187	2552	alticola	F
-69.80995	-32.85187	2552	alticola	M
-69.63432	-35.01203	1670	alticola	M
-69.91977	-34.84570	2093	alticola	F
-69.91977	-34.84570	2093	specularioides	F
-69.91977	-34.84570	2093	alticola	M
-69.62811	-36.76321	1891	alticola	M
-70.06362	-35.81625	1522	alticola	M
-68.38254	-16.18806	4307	alticola	M
-68.38254	-16.18806	4307	alticola	F
-68.35228	-16.12941	4374	alticola	M
-68.35228	-16.12941	4374	alticola	F

23419	REW-721	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23418	REW-723	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23417	REW-724	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23414	REW-727	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
	KGM-1278	14 Jun 2006	Perú	Junín	35 km SE Huaros
	KGM-1290	15 Jun 2006	Perú	Junín	ca Marcapomacocha
	KGM-1292	15 Jun 2006	Perú	Junín	ca Marcapomacocha
	KGM 1293	15 Jun 2006	Perú	Junín	ca Marcapomacocha
22755	KGM-1301	16 Jun 2006	Perú	Junín	ca Marcapomacocha
22753	KGM-1310	19 Jun 2006	Perú	Junín	Huarimarcán
22754	KGM-1319	21 Jun 2006	Perú	Pasco	24 km NE Paucartambo
22752	KGM-1359	3 Jul 2006	Perú	Ancash	Laguna Pelagato

-68.31933	-16.08057	4611	alticola	F
-68.31933	-16.08057	4611	alticola	M
-68.31933	-16.08057	4611	alticola	F
-68.31933	-16.08057	4611	alticola	M
-76.26123	-11.20559	4602	alticola	M
-76.17382	-11.24202	4218	alticola	M
-76.17382	-11.24202	4218	alticola	F
-76.17382	-11.24202	4218	alticola	M
-76.17382	-11.24202	4218	alticola	M
-76.23129	-11.05242	4605	alticola	M
-75.56150	-10.52204	4325	alticola	F
-77.81005	-8.17287	3976	alticola	F

Chapter 3:

Hemoglobin transcript abundance in a cDNA library from bone marrow of Crested Ducks (*Lophonetta specularioides*) in the Peruvian high Andes[1]

3.1 ABSTRACT.—Hemoglobins play a key role in oxygen transport. High-oxygen-affinity hemoglobins are adaptive in hypoxic environments. To better understand adaptation to high-altitude hypoxia, we extracted RNA from the bone marrow of six Crested Ducks (*Lophonetta specularioides*) inhabiting the central high Andes of Peru (4,218–4,605 m elevation) and sequenced >2,000 expressed sequence tags (EST) from a non-normalized complementary-DNA (cDNA) library. Overall, we identified 1,692 ESTs in the expression profile representing 462 different genes. Among those, the ESTs that occurred at the highest frequency were the αA (major) hemoglobin subunit (HBA2; 22.7%), leukocyte cell-derived chemotaxin 2 (LECT2; 10%), αD (minor) hemoglobin subunit (HBA1; 9.6%), beta defensins (DEFB; 6.3%), and the βA hemoglobin subunit (HBB; 3.7%). These results provide the first quantitative identification of gene expression in bone marrow of individuals inhabiting high-altitude regions and are in agreement with the known hemopoietic and immune function of this tissue. The EST sequences identified here will be useful for a variety of studies focusing on other nontraditional model

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[1] Bulgarella, M., N.C. Stewart, V.B. Fedorov, A.V. Moore, and K.G. McCracken. 2009. Hemoglobin transcript abundance in a cDNA library from bone marrow of Crested Ducks (*Lophonetta specularioides*) in the Peruvian high Andes. *The Auk* 126: 666–672.

organisms. Further studies of Crested Ducks and other highland taxa will be required to determine whether the αA and αD hemoglobin subunits, which contribute to distinct isoforms with potentially different oxygen-binding properties, are differentially expressed in lowland and highland populations.

3.2. INTRODUCTION

Tolerance to high-altitude hypoxia varies widely, and some organisms have evolved extraordinary capabilities to survive under limited oxygen availability (Hochachka and Lutz 2001). Hypoxia is among the most important ecological factors affecting survival in high-altitude regions, which typically are defined as elevations above 2,000 m (Hornbein and Schoene 2001), and highland organisms have developed a variety of physiological and molecular mechanisms to cope with life at high altitude (Hochachka and Somero 2002, Weber 2007, Storz and Moriyama 2008).

Two species of waterfowl, Bar-headed Goose (*Anser indicus*) and Andean Goose (*Chloephaga melanoptera*), have featured prominently in studies of high-altitude adaptation. Amino acid substitutions in two hemoglobin subunit genes (Pro→Ala-α119 and Leu→Ser-β55) produce similarly large effects by eliminating Van der Waals interactions on the same intersubunit contact, destabilizing the deoxy (T-state) structure and increasing the affinity of the major hemoglobin for oxygen (Perutz 1983, Jessen et al. 1991, Weber et al. 1993).

Most vertebrate hemoglobins (Hb) are tetrameric proteins composed of two α subunits and two β subunits. Birds express seven globin genes during development, in a variety of combinations. These include four embryonic genes (απ, βε, βρ, and βH) and three adult genes (αA, αD, and βA; Rowley and Ratcliffe 1988). Adult birds possess two hemoglobin components, one major isoform HbA (85% αA/βA) and one minor isoform HbD (15% αD/βA) (Borgese and Bertles 1965, Saha and Ghosh 1965). The major and

minor isoforms differ only in composition of the α chains (αA vs. αD); the β chains are identical (Weber et al. 1993). The mature erythrocytes of adult birds thus contain a mixture of functionally distinct hemoglobin isoforms with different biochemical properties and potentially different oxygen-binding properties (Hoffmann and Storz 2007). Several species of birds, however, have been shown to lack a minor hemoglobin isoform in blood assays (Godovac-Zimmermann and Braunitzer 1984, 1985; Oberthür et al. 1986). Recently, Hoffmann and Storz (2007) concluded that the αD subunit of amniote vertebrates arose via duplication of a gene with a larval–embryonic function in the ancestor of all tetrapods, which suggests that the αD subunit of the HbD isoform was pre-adapted for oxygen loading at low partial pressures of oxygen. HbD has higher oxygen affinity and cooperativity of oxygen binding than HbA (Cirotto and Geraci 1975, Baumann et al. 1984, Riggs 1998, Knapp et al. 1999). Therefore, regulatory adjustments altering the stoichiometric ratio of these two isoforms may modulate rates of oxygen flux in response to changes in metabolic demand (Hiebl et al. 1987, 1988).

Several recent studies have examined gene expression in humans native to high-altitude regions. Appenzeller et al. (2006) studied gene expression induced by hypoxia in Andean highlanders in Peru, and Rajput et al. (2006) studied expression of endothelin 1 in Himalayan natives. Fewer studies of gene expression have been performed on high-altitude non-human animals, and most such studies have focused on traditional model organisms. For example, Dolt et al. (2007) investigated hypoxia-induced transcriptional changes in the liver of mice, Zhang et al. (2007) reported expression patterns of hypoxia-inducible factor 1α (HIF1α) in Tibetan chicken (*Gallus gallus*) embryos, and D. P. Wang

et al. (2006) measured expression levels of HIF1α in Tibetan yak (*Bos grunniens*). Cheviron et al. (2008) applied transcriptomic profiling to the study of high-altitude adaptation in a natural population of non-model organisms. They studied differential gene expression in Rufous-collared Sparrows (*Zonotrichia capensis*) distributed along an altitudinal gradient in the Andes of Peru, where they observed substantial plasticity in the biochemical pathways associated with cold temperature and hypoxia compensation.

Expressed sequence tag (EST) analysis has been used extensively in large-scale complementary-DNA (cDNA) sequencing projects to identify differentially regulated genes (Brauch et al. 2005, Liu and Yang 2005). In addition, it provides quantitative information on the abundance of transcripts, as well as the possibility of identifying novel genes (Velculescu et al. 1995), and contributes to the development of polymerase chain reaction (PCR) primers for new loci for phylogenetic and phylogeographic studies (Backström et al. 2008). Several cDNA libraries have already been made for a small number of bird species, such as Wild Turkey (*Meleagris gallopavo*; Smith et al. 2000, Dranchak et al. 2003, Chaves et al. 2005), Zebra Finch (*Taeniopygia guttata*; Z. Wang et al. 2006b), House Finch (*Carpodacus mexicanus*; Z. Wang et al. 2006a), and Mallard (*Anas platyrhynchos*; Xia et al. 2007; reviewed by Bonneaud et al. 2008). Numerous other studies have analyzed gene-expression profiles in the Zebra Finch (Li et al. 2007, Slate et al. 2007). However, to our knowledge, this is the first cDNA library of a highland organism created from bone marrow, which is the principal site of hemopoiesis in adult birds. We quantified levels of gene expression in bone marrow of six Crested Ducks (*Lophonetta specularioides*) collected at 4,218–4,605 m elevation in the central high

Andes of Peru, using >2,000 expressed sequence tags (ESTs) from a non-normalized cDNA library. The present study is an important first step for studies of avian transcription profiling, which ultimately may be used to determine whether tissue-specific gene expression of highland taxa differs from lowland taxa, with higher expression of the high-oxygen-affinity hemoglobin isoforms predicted to occur in the highlands.

3.3 Methods

3.3.1 Sample collection.—We collected bone marrow from the epiphyses and diaphyses of leg and wing bones of six adult Crested Ducks from the high Andes of Peru in July 2006 at elevations of 4,218–4,605 m (Appendix 3.1). Tissues were harvested within 5 min of collection and stored in RNAlater, a stabilization reagent (Qiagen, Valencia, California) that allowed us to preserve the integrity of the RNA. After letting the buffer impregnate the tissues for 2–4 h, samples were stored in liquid nitrogen tanks in the field and –80°C laboratory freezers for long-term storage.

3.3.2 RNA isolation.—RNA was extracted from bone marrow using the RNeasy Mini Kit (Qiagen). Bone marrow was disrupted in liquid nitrogen and ground with a mortar and pestle into a fine powder. We added buffer RLT and homogenized the lysate by passing it at least five times through a blunt 20-gauge needle fitted to a syringe. Ethanol was then added to the lysate, promoting selective binding of RNA to the RNeasy membrane. The sample was applied to the RNeasy Mini spin column. Total RNA was

bound to the membrane, whereas contaminants were washed away, and RNA was eluted in RNase-free water. All bind, wash, and elution steps were performed in a microcentrifuge.

3.3.3 cDNA library construction.—A non-normalized directional cDNA library was created from the bone marrow of highland Crested Ducks using the Creator SMART cDNA Library Construction Kit (Clontech, Mountain View, California). The library was generated from wing and leg bone marrow from four males and two females. First-strand cDNA synthesis was performed using PowerScript Reverse Transcriptase. Second-strand cDNA synthesis was performed by primer extension. cDNA was digested by Sfi I enzyme. The cDNA molecules were fractioned by column chromatography to reduce the chance of cloning smaller inserts (<400 base pairs). cDNA was ligated to the Sfi I-digested, dephosphorylated pDNR-LIB vector provided with the kit. Recombinant plasmids were transformed into *Escherichia coli* after removing 1 μL of the ligation samples to mix with 20 μL ElectroMAX DH10B cells (Invitrogen, Carlsbad, California), and the mixture was electroporated by a Micropulser (BIO-RAD, Hercules, California). Products were resuspended and cultured at 37°C for 1 hr, in a shacking incubator at 225 rpm. An aliquot of the library was incubated on Luria Bertani (LB) agar plates in the presence of 30 μg mL^{-1} of chloramphenicol overnight.

3.3.4 Template preparation and sequencing.—cDNA clones were propagated for sequencing by transferring recombinant *E. coli* colonies to 96-well plates containing 200 μL LB + 20 μg mL^{-1} chloramphenicol per well. Bacteria were grown at 37°C overnight. PCR was performed directly on this cell suspension (1 μL cell suspension template, 25

μL PCR volume) by using universal M13 primers. Amplified cDNA inserts were sequenced from the 5′ end with the forward M13 primer by Agencourt Bioscience Corporation (Beverly, Massachusetts).

3.3.5 EST annotation.—Raw sequences were first screened by visual inspection, and the pDNR-LIB vector was trimmed. Each cDNA sequence was subjected to the BLASTn local alignment program (v.2.2.18; Altschul et al. 1990) against the nonredundant nucleotide database. Complementary-DNA identifications were assigned, based on high sequence identity to previously characterized genes submitted to the database; only assignment with a BLASTn score >150 and an expectation value $<10^{-5}$ were considered significant annotations, and cDNA sequences not meeting these criteria were omitted from the expression profiles and statistical analysis, as well as all sequences shorter than 120 base pairs (Brauch et al. 2005). ESTs that exceeded these criteria were tabulated to determine the total number of each gene expressed in the bone marrow of highland Crested Ducks. Percent gene expression was computed by summing the number of ESTs matching the particular gene and dividing the sum by the total number of ESTs that matched known genes. We considered an EST a novel gene if it did not match any genes of known function in GenBank but matched numerous ESTs, which provided evidence that it represented a real transcript (Velculescu et al. 1995). One representative EST from every gene identified was submitted to the GenBank dbEST (accession numbers GO307587–GO308024).

3.4 Results

The total number of cDNAs sequenced was 2,128. Among these, 234 yielded poor sequence read, no insert, or insufficient BLASTn alignment; 202 sequences were not identified in GenBank, potentially representing novel genes. Overall, we identified 462 different genes in the expression profile. Because the library was neither subtracted nor normalized, the number of clones from an individual gene may approximate the expression level of these genes in Crested Duck bone marrow (see also Xia et al. 2007). High redundancy of a specific cDNA sequence among ESTs is likely correlated with a higher expression level of that gene; therefore, the ESTs appearing frequently in the expression profile may be regarded as "highly expressed genes" (Liu and Yang 2005). In total, 1,692 unique GenBank identifications were made from a total of 1,963 high-quality sequences. A summary of the distributions of all cDNA data is shown in Table 3.1.

The complete digital expression profile from this Crested Duck cDNA library is available upon request and at our website (see Acknowledgments). To establish a baseline for gene expression in duck bone marrow, we identified the 20 most abundant ESTs (Table 3.2). Our analysis shows that mRNA coding for the three subunits (αA, αD, βA) of the adult hemoglobin protein isoforms were among the five most common ESTs in the profile. The transcripts included hemoglobins (36%), together with leukocyte cell-derived chemotaxin 2 (LECT2; 10%), beta defensins (DEFB; 6.3%), mitochondrial cytochrome oxidase I (MT-CO1; 2.4%), immunoglobulin J (IGJ; 2.3%), ubiquitin C (UBC; 1.2%), and mitochondrial ATP synthase 6 (MT-ATP6) and cytochrome-*c* oxidase

III (MT-CO3; 1%), among others. Considering only hemoglobins, the most frequently expressed subunit was αA (HBA2; 63%), the αD (HBA1) subunit constituted 26.7%, and the βA (HBB) subunit represented 10.3% of the total hemoglobin expressed. We identified a total of 1,692 ESTs coding for 462 different genes in the profile. However, the gene number represented by these ESTs is actually <462. This is attributable to the fact that the ESTs corresponding to different regions of the same gene or paralogous subunits of a gene that belongs to duplicated gene families do not assemble together in the same contig. For example, in the chicken genome, 13 beta defensin genes are densely clustered within 86 Kb distance on chromosome 3q3.5–q3.7 (Xiao et al. 2004). Despite belonging to the same gene cluster, the beta defensin, beta defensin 1, and beta defensin 3 genes shown in Table 2 do not form a contig. Furthermore, alternatively spliced forms of a single gene can group in a different contig (Liu and Yang 2005). Following the gene ontology (GO) website, we classified the most redundant transcripts according to their function and biological process (Table 3.3). GO associations organize genes into their respective biological niches and greatly reduce data complexity, revealing the most distinguishing characteristics of each group (Xia et al. 2007).

3.5 Discussion

Red bone marrow produces red blood cells, white blood cells, and platelets through a process called hemopoiesis (Tortora and Derrickson 2006). It consists of developing blood cells, adipocytes, fibroblasts, and macrophages within a network of reticular fibers.

It is present in developing bones of the fetus and in some adult bones, such as the pelvis, ribs, breastbone, vertebrae, skull, and ends of the bones of the arm and thigh. Yellow bone marrow consists mainly of adipose cells, which store triglycerides. Red bone marrow becomes the primary site of hemopoiesis in the last three months before birth and continues as the main source of blood cells after birth and throughout life (Tortora and Derrickson 2006). Red blood cells or erythrocytes contain the oxygen-carrying protein hemoglobin. Normal human blood has a hemoglobin concentration of 15 g per 100 mL (Hardison 1998). In birds of many species, hemoglobin occupies one-third of the red-blood-cell volume (Campbell 1995). Furthermore, hemoglobins are found in virtually all kingdoms, including eubacteria, unicellular eukaryotes, plants, and animals (Hardison 1998). Leukocyte cell-derived chemotaxin 2 functions in bone development and chemotaxis; genes encoding leukocyte markers can be expressed for generation of antibodies to discriminate between immune cell types (Xia et al. 2007). Beta defensins are cysteine-rich, cationic peptides with *in vitro* bactericidal effects for both avian and human bacterial pathogens (Ganz 2003). There are seven beta defensin genes predominantly expressed in bone marrow and the respiratory tract in chickens. However, the functional significance of these genes during inflammation and infection remains unknown (Xiao et al. 2004). Cytochrome oxidases are involved in aerobic respiration and oxidative phosphorylation. Immunoglobulins are glycoproteins with an antibody activity found in the blood, lymph, and vascularized tissues of all the jawed vertebrates (Davison et al. 2008).

Our EST data provide both sequence information and a general summary of the gene-expression profile in highland Crested Duck bone marrow. As expected, our results revealed ESTs for many genes related to oxygen transport and immunological function, therefore faithfully reflecting the role of bone marrow as a hemopoietic and immunological tissue.

Furthermore, 70% of the total level of α hemoglobin expressed corresponded to the αA subunit, and 30% to the αD subunit, in accordance with the known major and minor HbA and HbD components of bird blood (Borgese and Bertles 1965, Saha and Ghosh 1965). The low levels of βA globin subunit may be attributable to differential temporal hemoglobin synthesis. In the crustacean *Daphnia magna*, the strategy to optimize oxygen transport to the tissues when exposed to hypoxia includes the differential synthesis of hemoglobin subunits of increased oxygen affinity (Zeis et al. 2003). Hemoglobin genes are thus regulated independently, allowing for differential expression. In vertebrates, hypoxic induction is regulated by a hypoxia-inducible transcription factor binding to oxygen-responsive elements on the DNA (for a review see Zhu et al. 2002). Zhang et al. (2007) analyzed the expression pattern of hypoxia-inducible transcription factor 1α in Tibetan Chickens. Furthermore, Cheviron et al. (2008) reported a high degree of plasticity in the gene-expression patterns of Rufous-collared Sparrows inhabiting a steep elevational gradient in the Andes.

We do not know whether differential expression of the major (HbA) and minor hemoglobin (HbD) isoforms plays an important role in highland adaptation in species like the Crested Duck. Cirotto and Geraci (1975), Baumann et al. (1984), Riggs (1998), and

Knapp et al. (1999) reported that HbD has higher O_2 affinity than HbA, but data are not yet available for Crested Ducks.

Crested Ducks comprise two subspecies: *Lophonetta s. specularioides* is endemic to Patagonia and the Falkland Islands; *L. s. alticola* inhabits the high Andes from central Peru to southern Mendoza, Argentina, and Talca, Chile (Navas and Bo 1998, Bulgarella et al. 2007). The two subspecies inhabit different elevational environments, ranging from sea level in Patagonia (*specularioides*) to 5,000 m in the central high Andes (*alticola*). Differential expression of major and minor hemoglobins may exist between highland and lowland populations of Crested Ducks if the αA and αD subunits differ in their intrinsic O_2 affinity or if individual αA or αD subunits coded by different alleles differ in their O_2 affinity because of specific amino acid mutations. Highland Crested Ducks, in particular, would be expected to have higher expression levels of the minor HbD isoform if it possesses higher O_2 affinity. Physiological and quantitative molecular studies of gene expression in the lowlands and highlands should be performed to further explore adaptations to high-altitude hypoxia in birds.

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Table 3.1 Composition of Crested Duck bone marrow cDNA.

Total sequenced cDNA	2,128	Percentage (%)
No insert, poor sequence read, insufficient BLASTn alignment	234	
No match in GenBank	202	
Mitochondrial DNA, rRNA	165	9.8
ESTs representing hemoglobins (total)	611	36.1
αA hemoglobin subunit (HBA2)	385	
αD hemoglobin subunit (HBA1)	163	
βA hemoglobin subunit (HBB)	63	
ESTs representing other proteins	916	54.1

Table 3.2 The 20 most frequent transcripts in Crested Duck bone marrow.

Gene	Counts	%
αA hemoglobin subunit (HBA2)	385	22.75
Leukocyte cell-derived chemotaxin 2 (LECT2)	171	10.11
αD hemoglobin subunit (HBA1)	163	9.63
Beta defensin (DEFB)	76	4.49
βA hemoglobin subunit (HBB)	63	3.72
Cytochrome oxidase I (MT-CO1)	41	2.42
Immunoglobulin J (IGJ)	39	2.30
Ubiquitin C (UBC)	22	1.30
Beta defensin 3 (DEFB3)	20	1.18
ATP synthase 6 and cytochrome-*c* oxidase III (MT-CO3)	18	1.06
mRNA for hypothetical proteins	18	1.06
Cytochrome-*c* oxidase II (MT-CO2)	17	1.00
Predicted: hypothetical loci mRNA	13	0.77
Beta-defensin 1 (DEFB1)	12	0.71
Heat shock protein 90 alpha (HSP90A)	9	0.53
Predicted: similar to cysteine proteinase inhibitor	8	0.47
Predicted: similar to transglutaminase 3 (TGM3)	8	0.47
Lymphocyte antigen 86 (LY86)	7	0.41
H5 histone	7	0.41
BAC clone from chromosome Z	7	0.41

Table 3.3 Gene Ontology classification for the most common protein-coding transcripts found in bone marrow of Crested Ducks.

Gene name	Biological process	Molecular function	Cellular component
Hemoglobins	Hemopoiesis Response to hypoxia Oxygen transport	Oxygen transport activity Oxygen binding	Hemoglobin complex
Leukocyte cell-derived chemotaxin 2	Chemotaxis Skeletal development	–	Cytoplasm Extracellular space
Beta defensins	Chemotaxis G protein coupled receptor protein signaling pathway Innate immune response	–	–
Immunoglobulin J	Immune response	Antigen binding	Extracellular region
Ubiquitin C	Regulation of transcription Response to heat, stress ATP-dependent proteolysis Phagocytosis, engulfment	Protein binding	Cytoplasm Nucleus
Cytochrome oxidases	Transfer of electrons from cytochrome-*c* to oxygen	Cytochrome-*c* oxidase activity	Mitochondrial inner membrane
Heat shock protein 90 alpha	Muscle thick filament assembly Myofibril assembly	Protein binding	Z disc
Lymphocyte antigen 86	Apoptosis Cell proliferation Immune response	Protein binding	Plasma membrane

Appendix 3.1 Localities for Crested Ducks included in the present study.

Field catalogue number[a]	Date	Locality	Longitude	Latitude	Elevation (m)
KGM 1278	14 June 2006	Junín, 35 km SE Huaros	76.26123 W	11.20559 S	4,602
KGM 1288, 1290, 1292, 1293	15 June 2006	Junín, Marcapomacocha	76.17382 W	11.24202 S	4,218
KGM 1310	19 June 2006	Junín, Huarimarcán	76.23129 W	11.05242 S	4,605

[a] University of Alaska Museum.

Chapter 4:

Multilocus coalescent analyses reveal hemoglobin differentiation between high- and low-elevation populations of crested ducks (*Lophonetta specularioides*)[1]

4.1 Abstract

Hypobaric hypoxia is a key factor determining survival at high elevation, and hemoglobin proteins are probable targets of selection. We examined the population genetics of the three adult hemoglobins of the crested duck (*Lophonetta specularioides*). Its two recognized subspecies inhabit highland and lowland regions, respectively. We also included six autosomal introns and mtDNA for independent population genetic contrasts. Four unique amino acid replacements were found in the globins that differed in frequency between highland and lowland populations with significantly elevated F_{ST} values. Coalescent analyses suggested that gene flow is restricted for hemoglobin alleles and mtDNA between highland and lowland populations, whereas some level of gene flow was detected for the introns. Divergent selection with local adaptation favoring different homozygous genotypes is consistent with the pattern observed for the single polymorphism on the βA subunit. Overdominance likely shaped the evolution of high-altitude function in the α subunits. Eight individuals collected at intermediate elevations

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[1] Bulgarella, M., J.L. Peters, T.H. Valqui, R.E. Wilson, and K.G. McCracken. Multilocus coalescent analyses reveal hemoglobin differentiation between high- and low-elevation populations of crested ducks (*Lophonetta specularioides*). Prepared for submission to *Evolution*.

(1,522–2,552 m, Mendoza, Argentina) where the two subspecies intergrade, revealed unique combinations of hemoglobin genotypes not found elsewhere. Mendoza individuals also had intermediate morphology and admixed nuclear DNA. A bounded hybrid-superiority model may explain the pattern and frequency of genotypes we observed in this region.

4.2 Introduction

High-elevation environments present a number of physiological challenges for endothermic animals, including colder temperatures, increased desiccation, and higher atmospheric radiation (Monge and León-Velarde 1991, Rezende et al. 2005). One of the most noticeable high-elevation effects is hypoxia. The partial pressure of oxygen (P_{O2}) decreases with elevation by approximately ten percent per thousand meters. At elevations such as 4,000 m in the Andean or Himalayan plateaus the P_{O2} of inspired air is 60% of that at sea level (Beall 2007).

Animals vary in their capabilities to withstand hypobaric hypoxia. Among vertebrates, birds are particularly well adapted for maintaining oxygen supplies to the tissues. For example, house sparrows (*Passer domesticus*) remain alert and exhibit normal behavior at atmospheric pressures equivalent to 6,100 m, whereas mice become comatose (Tucker 1968). In addition, numerous species of birds undertake high-elevation flights or migrations. A Rüppell's griffon (*Gyps rueppellii*) collided with a jetliner at 11,300 m (Laybourne 1974), and bar-headed geese (*Anser indicus*) migrate over the Himalayas at 9,000 m of altitude (Swan 1961, 1970, Scott and Milsom 2007). Flying at high altitudes, where P_{O2} is reduced, is particularly demanding because O_2 consumption during flight can increase as much as twenty-fold (Bernstein 1989).

How birds and other organisms have adapted to high-elevation regions continues to be an important question for physiologists and evolutionary biologists. All species of birds possess crosscurrent arrangements of gas and blood flow that make the avian lung more efficient than the alveolar lungs of mammals (Piiper 1989, Scheid 1979). Bird

muscles possess higher venous oxygen tension and increased densities of capillaries, mitochondria, and myoglobin than mammalian red muscle tissue (Grubb 1981, Faraci 1991). Additionally, other likely adaptive traits such as higher red blood cell counts, larger lung volume, and blunted hypoxic ventilatory response have been identified in vertebrates residing at high elevations (Beall 2003). Furthermore, biochemical adaptations in O_2 binding proteins such as hemoglobin (Hb) and myoglobin may facilitate either O_2 uptake at the lungs or O_2 release at the tissues. Hemoglobin binds oxygen at the lungs and delivers it to the respiring tissues (Hardison 2001). In the case of hemoglobin, examples include increases in Hb concentration in the blood, changes in Hb-O_2 affinity, and changes in allosteric properties (Hochachka and Somero 2002).

Several recent studies have shown that genetically based adaptations that increase the O_2-binding properties of hemoglobins play an important role in physiological adaptation to high-elevation hypoxia (Storz et al. 2007, Weber 2007, Storz and Moriyama 2008). Most vertebrate hemoglobins are tetrameric proteins composed of two α polypeptide subunits and two homologous β subunits each bind O_2 cooperatively and reversibly at their hemes (Perutz 1983, 1989). The α and β subunits are encoded by two different sets of duplicated genes located on different chromosomes. In birds such as chicken (*Gallus gallus*) and ducks there are seven different hemoglobin genes, four of which are expressed at embryonic or juvenile states of the life cycle ($\alpha\pi$, $\beta\varepsilon$, $\beta\rho$, βH) and three that are expressed in adult birds (αD, αA, βA; Rowley and Ratcliffe 1988, Bulgarella et al. 2009). Adult birds thus possess two different hemoglobin isoforms, a major HbA (αA/βA) and a minor HbD (αD/βA) that share the same β-chain but differ in

their O_2 binding properties due to differences in the coding sequences of the αA and αD subunits (Weber et al. 1993, Weber and Fago 2004). In the Muscovy duck (*Cairina moschata*), the two adult α globins are separated by 2.2 kb of DNA, encoded by the same DNA strand, and transcribed in the 5'-αD-αA-3' direction (Niessing et al. 1982). The HbD isoform has higher O_2 affinity and cooperativity of O_2-binding than HbA (Cirotto and Geraci 1975, Baumann et al. 1984, Riggs 1998, Knapp et al. 1999). Circulating red blood cells contain a mixture of isoHbs with different O_2-binding affinities (Hiebl et al. 1987, 1988, Weber et al. 1988). Regulatory adjustments altering the stoichiometric ratio of these two isoforms may modulate rates of O_2 flux in response to changes in metabolic demand or to different environments. Hemoglobin-O_2 affinity is also strongly influenced by concentrations of allosteric effectors such as Cl^-, H^+, CO_2, and organic phosphates within red blood cells. In birds, inositolpentaphosphate (IPP) is the most potent allosteric effector. In avian red blood cells, IPP and other allosteric ligands exert their regulatory effects by preferentially binding to the low-affinity deoxygenated (T-state) conformation of the hemoglobin tetramer. Hemoglobins have been shown to be under strong selection in high-elevation environments in a variety of taxa (Snyder 1981, Chappell et al. 1988, McCracken et al. 2009a, 2009b, Storz et al. 2007, 2009, Storz and Hoekstra 2007).

In this study, we assessed population genetic structure between highland and lowland populations and evaluated evidence for local adaptation in the hemoglobin genes of the crested duck (*Lophonetta specularioides*), a monotypic genus of dabbling duck (Anatinae) endemic to South America. We compared the three adult hemoglobin genes (αA, αD, and βA) that are known to be expressed in the bone marrow of this species

(Bulgarella et al. 2009) to a series of autosomal reference loci and the mtDNA control region using interlocus contrasts.

4.2.1 DETECTING DIFFERENTIALLY SELECTED LOCI

Interlocus contrasts have been used extensively to detect genomic outliers, putative candidate loci, and polymorphisms within loci that have evolved in response to differential selection (Beaumont 2005, Storz 2005). However, measures of population differentiation such as fixation indices like F_{ST} or Φ_{ST} may or may not always be useful for such purposes. Outliers may arise stochastically or be imperceptible among background sequence variation (Nosil et al. 2008). For example, differentially selected loci may be difficult to detect if populations are geographically structured at most loci (i.e., Φ_{ST} is generally elevated at sites across the genome) or if there is extensive linkage disequilibrium. Fixation indices such as Φ_{ST} may also be less useful for populations that have high diversity at the candidate locus. For example, if most individuals in a population are heterozygous for a particular combination of alleles, Φ_{ST} values, which are bounded by zero and one by definition, will be intermediate in magnitude, and even smaller if selection strongly favored different homozygous genotypic classes in opposite environments.

An alternative approach for detecting differentially selected loci is to use coalescent models that incorporate the time since divergence and other aspects of population history, such as the rate at which alleles move between populations inhabiting different environments. This approach has several advantages compared to descriptive

measures of population differentiation because it incorporates genetic stochasticity by sampling from the range of genealogies that are consistent with the data (Nielsen and Wakeley 2001; Hey and Nielsen 2004). In the case of populations inhabiting different environments, local selection may lower the effective migration rate, because migrants may carry locally adapted alleles to unfavorable environments, and these alleles will be disfavored by selection (Barton 1979, Bengtsson 1985, Barton and Bengtsson 1986). As a result of the reduced effective migration rate at the divergently selected locus, the mean coalescence time (time to most recent common ancestor; $TMRCA_T$) of two alleles sampled randomly from the total population will be higher than the mean coalescence time of two alleles sampled from the same subpopulation ($TMRCA_S$). Sites closely linked to the selected locus may also exhibit an elevated level of divergence relative to the genome-wide average due to hitchhiking (Charlesworth et al. 1997, Gillespie 2000). Using coalescent methods to compare TMRCA and divergence times between candidate loci and putatively neutral loci can be a powerful way to test for deviations from the standard neutral model.

4.2.2 STUDY SPECIES

Crested duck inhabits different elevational environments. Its two recognized subspecies are *L. s. specularioides*, which inhabits low elevation habitats in the southern Andean regions of Patagonia and the Falkland Islands, and *L. s. alticola*, which occurs throughout the Andean highlands up to approximately 4,800 m elevation from northern Peru to central Argentina (Fjeldså and Krabbe 1990, Schulenberg et al. 2007). The two

subspecies can be distinguished morphologically. *L. s. specularioides* has a bright red iris, smaller body size (wing chord < 280 mm), and darker, more mottled plumage than *L. s. alticola*, which is larger (wing chord > 280 mm) and possesses a yellow-orange iris (Phillips 1922–26, Johnsgard 1978, Kear 2005). The two subspecies intergrade in a zone of intermediate elevational habitats in Mendoza, Argentina, and Talca, Chile (Navas and Bo 1998, Bulgarella et al. 2007). Crested duck thus is an excellent species for investigating how changes in the amino acid sequence of the αA, αD, and βA hemoglobin genes might confer resistance to hypoxia in different elevational zones.

4.3 Methods

4.3.1 SPECIMEN COLLECTION

The Andean Cordillera comprises the long chain of mountain ranges and volcanoes that parallel the western margin of South America. The central Andes, including the Altiplano, is the widest part of the cordillera. It consists of a series of high plateaus and inter-montane valleys that extend from Cajamarca, Peru, to Catamarca, Argentina, and Copiapo, Chile. South of Catamarca, the Andes narrow, lose elevation, and gradually transition to the southern Andes of Patagonia. As a result, most wetlands and grasslands that are suitable for waterfowl occur at elevations > 3,000 meters in the central Andes, whereas most waterfowl habitat in the southern Andes occurs at elevations < 1,500 meters.

We collected crested ducks throughout their range in the Andes. Eighty specimens were collected, including 49 *L. s. alticola* from high-elevation sites in Peru, Bolivia, and

northwestern Argentina (3,338–4,611 m) and 23 *L. s. specularioides* from low elevation sites in Patagonia and the Falkland Islands (0–934 m). Eight additional *L. s. alticola* specimens were collected from five intermediate elevation sites in Mendoza, Argentina (1,522–2,552 m; Fig. 4.1, Appendix 4.1). Each individual was identified to subspecies using previously published analyses of plumage and morphology (Bulgarella et al. 2007).

4.3.2 DNA SEQUENCING

We sequenced the three adult hemoglobin genes (αA, αD, and βA) and compared them to six reference loci located on different chromosomal linkage groups. The complete coding regions of the αA and βA hemoglobin subunits (677 bp and 1581 bp, respectively) and the 5' end of the αD subunit (435 bp spanning exon 1, intron 1, and part of exon 2) were amplified using PCR and sequenced using standard protocols described by McCracken et al. (2009c). The mtDNA control region was sequenced using the overlapping primer pairs L78-H774 and L736-H1252 or H1530 (Sorenson and Fleischer 1996, Sorenson et al. 1999, McCracken and Sorenson 2005). Six intron sequences, ranging from 246 to 353 bp in length, were also sequenced, and those included ornithine decarboxylase intron 5 (ODC1), alpha enolase intron 8 (ENO1), beta fibrinogen intron 7 (FGB), N-methyl-D-aspartate-1-glutamate receptor intron 11 (GRIN1), phosphoenolpyruvate carboxykinase intron 9 (PCK1), and lamin A intron 3 (LMNA). Primers for each locus were developed specifically for ducks, and all intron loci were chosen blind to levels of polymorphism (McCracken and Sorenson 2005, McCracken et al. 2009c, 2010).

Sequences from opposite strands were reconciled and verified for accuracy using Sequencher v.4.7 (Gene Codes, Ann Arbor, MI). Double peaks indicating the presence of two alleles were coded with IUPAC degeneracy codes and treated as polymorphisms. Indels were resolved by comparing forward and reverse strands, using the unambiguous 5' end of one strand to edit the ambiguous 3' end of the other strand to determine the length of the indel (Peters et al. 2007). Gaps resulting in shifted peaks in the chromatograms were coded as a fifth character state.

The gametic phases of sequences that were heterozygous at two or more nucleotide positions were determined using PHASE v.2.1.1 (Stephens et al. 2001). PHASE uses a Bayesian method to infer haplotypes from diploid genotypic data while incorporating recombination and the decay of linkage disequilibrium with distance. We first analyzed the diploid consensus sequences of each individual using the default software values followed by 1,000 burn-in and 1,000 sampling iterations. The PHASE algorithm was run five times from different starting points, and the results with the best overall goodness of fit were selected. For individuals with allele pair probabilities < 80%, we designed allele-specific primers to selectively amplify one allele. The resulting haploid allele sequence was then subtracted from the consensus sequence to obtain the gametic phase of the second allele. Each data set was analyzed five more times using PHASE with the newly resolved alleles defined as 'known' alleles. PHASE analyses and allele-specific priming were performed for the complete αA and αD hemoglobin sequences and each of the six autosomal introns. For the βA subunit, we inferred the gametic phase of a 596 bp segment of spanning intron 1, exon 2, and part of intron 2 that

had no detectable recombination as determined using the four-gametes test (Hudson and Kaplan 1985). Overall, the gametic phases of 97.9% (n = 705) of the 720 individual autosomal sequences were identified experimentally or with > 95% posterior probability. All sequences were aligned by eye using SeAl v.2.0a11 (Rambaut 2007) and archived in GenBank (accession numbers GQ271065-GQ271144, GQ271804-GQ271883, GQ268964-GQ269043, GQ269044-GQ269123, GQ269124-GQ269203, GQ269204-GQ269283, GQ269284-GQ269363, HM063481-HM063503).

4.3.3 ANALYSES OF POPULATION DIFFERENTIATION

To determine whether lowland and highland populations of crested ducks are genetically differentiated, we compared Φ_{ST} between *L. s. specularioides* and *L. s. alticola* for each locus. We also performed the Mann-Whitney U test to compare the mean Φ_{ST} for the globins versus the mean Φ_{ST} for the introns. F_{ST} for each non-synonymous (amino acid) polymorphism in the αD, αA, and βA hemoglobin subunits was also calculated. Additionally, we tested Hardy-Weinberg equilibrium to determine if there was a significant excess or deficit of heterozygotes for any given locus, which might be interpreted as divergent selection acting on alleles that were overrepresented in one or the other population. All estimates of linkage disequilibrium, F_{ST} and Φ_{ST} were performed using the Tamura-Nei (1993) nucleotide substitution model in Arlequin v.3.01 (Excoffier et al. 2005). Allelic richness standardized to the smallest sample size (n = 46 alleles), was calculated using the software RAREFAC (Petit et al. 1998) and then compared between highland and lowland populations using a paired t-test. The eight individuals from

Mendoza were excluded from these analyses. Allelic networks for each locus were illustrated using the median-joining algorithm in NETWORK v.4.1 (Bandelt et al. 1999) and included the Mendoza specimens.

In addition to the basic summary statistics described above, we also used STRUCTURE v.2.2 (Pritchard et al. 2000) to examine population differentiation. STRUCTURE uses a Bayesian method to assign individuals to populations by maximizing Hardy-Weinberg equilibrium and minimizing linkage disequilibrium. We performed two STRUCTURE analyses; the first included only the six autosomal introns, and the second included the six introns plus the αA, αD, and βA hemoglobin genes (nine loci in total, mtDNA was not included in the analysis; see below). The eight individuals from Mendoza were included in this analysis, and no *a priori* information describing specimen locality was provided. We used the admixture model with independent allele frequencies, 10,000 generations of burn-in and 20,000 generations of sampling. The optimum number of crested duck populations was identified using the Δk method (Evanno et al. 2005). To determine the number of populations (k), we estimated ln Pr(X|k) for $k = 1$ to $k = 5$. Using the value of k with the highest ln Pr(X|k), we assigned individuals to the inferred populations.

4.3.4 COALESCENT ANALYSES

We compared the three hemoglobin genes to the six reference loci using a two-population isolation-with-migration (IM, Hey and Nielsen 2004) analysis that allows for both divergence and gene flow. We estimated indices of effective population size (Θ),

immigration rates (m), time since population divergence (t), and TMRCA for each locus. The software IM implements a Bayesian MCMC method to fit the data to a coalescent model. We estimated six population parameters scaled to the neutral mutation rate, μ: Θ_H ($4N_e\mu$ for highland crested ducks), Θ_L ($4N_e\mu$ for lowland crested ducks), Θ_A ($4N_e\mu$ for the ancestral population at the time of divergence), t (Tμ, where T is the time since divergence in years before the present), M_L (m_L/μ, where m is the rate of migration into the lowlands from highlands), and M_H (m_H/μ, the rate of migration into the highlands from the lowlands). The eight individuals collected from Mendoza were excluded from this analysis (see results below).

IM assumes that the loci are selectively neutral with no intralocus recombination. Therefore, we tested for recombination within each nuclear locus using a four-gametes test in DNAsp v.4.10 (Rozas et al. 2003) and included sequence data from the largest independently segregating block consistent with no recombination. For the hemoglobin genes we included the longest fragment consistent with no recombination that included all of the non-synonymous amino acid replacements that we observed; αD hemoglobin subunit positions 108–441, αA hemoglobin subunit positions 1–204, and βA subunit positions 140–735. GRIN1 and LMNA were truncated to the 5'-end positions 1–239 and positions 1–195, respectively. The remaining nuclear loci had no detectable recombination and therefore the full sequences were included in the analysis.

For mtDNA, which is haploid and maternally inherited, we defined the inheritance scalar to be 0.25 and used the HKY (Hasegawa et al. 1985) model of mutation. For nuclear loci, we defined the scalar to be 1.0 because they are biparentally

inherited, and we used an infinite-sites model of substitution. IM was first run with wide priors to set appropriate upper bounds for each parameter. These runs were then repeated using uniform priors that encompassed the full posterior distribution of each parameter from the preliminary runs and were therefore assumed to be uninformative. However, estimates of t sometimes contained distinct peaks, but the tails were flat and did not approach zero. In these cases, we defined an upper bound based on preliminary runs by assuming that time since divergence could not be older than TMRCA (Peters et al. 2007). Averaging the posterior distribution of TMRCA for the six nuclear introns, we used the upper 90% highest posterior density (HPD) of TMRCA as the upper bound for t (upper bound = 0.85). The priors encompassed the full posterior distributions of all other parameters. We repeated runs with a burn-in of at least 200,000 steps. To assess convergence, we monitored autocorrelations and effective sample sizes (ESS) for each parameter throughout the run (Hey and Nielsen 2004). The run was continued until the smallest ESS was at least 100 (see Hey 2005). We ran the analysis a second time with a different random number seed to be sure that different runs converged on the same parameter estimates. All runs included 10 heated chains using the default heating parameters. Finally, to get an accurate estimate of TMRCA, we analyzed each intron locus independently, because IM calculates a mutation rate scalar based on differences among loci for Θ. Therefore, if a locus has a high level of polymorphisms, then IM will assume that this is explained by a higher substitution rate than lower diversity loci.

To convert IM parameter estimates to biologically informative values, estimates of generation time (G) and mutation rate (μ per locus) are necessary. We used the control

region mutation rate calibrated by Peters et al. (2005) of 4.8 x 10^{-8} substitutions/site/year (s/s/y) for the mtDNA and calibrated rates for nuclear loci based on the goose-duck split (see Peters et al. 2007, 2008). The mean genetic distance between the snow goose (*Anser caerulescens*) sequence and resolved alleles for crested duck was divided by the mid-point of the Oligocene (2 x 30.5 million years). The geometric mean of substitution rates averaged for the six introns was 2.72 x 10^{-9} substitutions/site/year.

4.3.5 RECOMBINATION RATES AND GENETIC LINKAGE BETWEEN THE αA AND αD GLOBIN GENES

The α and β globin gene clusters have been shown to exhibit consistently elevated rates of recombination in a wide diversity of taxa (Chakravarti et al. 1984, Storz et al. 2007, McCracken et al. 2009a). Therefore we used the software LAMARC v.2.1 (Kuhner 2006) to estimate the overall recombination rate (r) for each locus, which is the ratio (ρ/μ) between per-site recombination rate and per-site mutation rate. The upper and lower limits for r were set to 0 and 10, respectively. In LAMARC, a recombination rate of 1 means that recombination is equally likely to occur as mutation, whereas a recombination rate of 0 means that no recombination was detectable. The eight individuals collected from Mendoza were excluded from the LAMARC analysis.

The αD and αA hemoglobin loci are physically linked on chromosome 14 and separated by approximately 2.2 kb in the Muscovy duck (Niessing et al. 1982). We examined evidence for gene conversion between the αD and αA subunits. Gene conversion plays an important role in the evolution of multigene families, as it brings

about the exchange of genetic material between related sequences (Schimenti 1994; Posada et al. 2002). It has been shown to be a frequent mechanism of evolutionary change in globins and can act both to homogenize genes through concerted evolution and to introduce novelty among homologous genes (Aguileta et al. 2004, Storz et al. 2007). Thus, we tested for gene conversion between the two paralogous α subunits using the algorithm developed by Betran et al. (1997) implemented in DNAsp v.4.10.

4.3.6 STRUCTURAL ANALYSIS OF THE BETA SUBUNIT

The position of the amino acid replacement in the beta subunits of the HbA isoform (αA/βA) of crested ducks was located on the oxy (R-state) crystal structure of greylag goose (*Anser anser*) hemoglobin (Liang et al. 2001a; Protein Data Bank 1FAW). The protein structure was illustrated using Cn3D v.4.1 (National Institute of Health, Bethesda, MD). The βA subunit positions Valβ1, Hisβ2, Lysβ82, Argβ104, Argβ135, Hisβ139, Argβ143, Lysβ144, and Hisβ146 comprise the first IPP-binding site (Zhang et al. 1996, Wang et al. 2000, Liang et al. 2001a,b, Liu et al. 2001).

4.4 Results

4.4.1 POPULATION DIFFERENTIATION

We found strong evidence of population differentiation between highland and lowland populations. Φ_{ST} values for the three globins, mtDNA, and the six introns ranged from 0.02 to 0.87 and were significantly different between populations for most loci (Table 4.1).

The mtDNA control region (Φ_{ST} = 0.85) yielded two reciprocally monophyletic clades corresponding to the lowland subspecies (*L. s. specularioides*) and the highland subspecies (*L. s. alticola*; Fig. 4.2A). All eight specimens collected in Mendoza, Argentina, possessed *L. s. alticola* haplotypes. Three *L. s. specularioides* from the Falkland Islands possessed a unique haplotype that was not shared with *L. s. specularioides* individuals from mainland Argentina (Fig. 4.2A).

In contrast to the mtDNA, most intron alleles, including the most common haplotypes, were shared between subspecies (Fig 4.2B). Φ_{ST} values for the six autosomal introns varied from a minimum of 0.02 in GRIN1 to a maximum of 0.44 in ODC1. Allelic richness did not differ between the highlands and the lowlands. An average of 4.4 different alleles was expected per locus in a random sample of 46 alleles (AR_{46}) from the highlands compared to 3.8 different alleles from the lowlands. Among intron loci, the differences were not significant (paired *t*-test, $t = 0.51$, df = 5, $P = 0.63$). No linkage disequilibrium was found between the six reference loci, confirming that these loci are unlinked (Ps > 0.99).

Using the six introns, the STRUCTURE analysis with the maximum likelihood (lnL = –2189.0) and greatest value of Δk was for $k = 2$ populations. Excluding the eight Mendoza specimens, the mean posterior probabilities of assignment to the highland population (> 3,000 meters) for *L. s. alticola* was 0.86 ± 0.18 and varied from 0.19 to 0.98. For *L. s. specularioides*, the mean posterior probability of assignment to the lowland population was 0.93 ± 0.06 and ranged from 0.77 to 0.99 (Fig. 4.3A). The eight individuals collected at 1,522–2,552 m of elevation and between 32.8–35.8°S in the

province of Mendoza (shown in grey in Fig. 4.3) exhibited the full range of posterior probabilities to be assigned to the highland population (0.05–0.97).

4.4.2 HEMOGLOBIN GENE DIFFERENTIATION

Φ_{ST} for the βA hemoglobin subunit (0.87) was higher than for any other locus. Φ_{ST} values for αA (0.23) and αD (0.33) hemoglobins were higher than for five of the six intron loci (Table 4.1). Furthermore, the comparison of the mean Φ_{ST} for the globins versus the mean Φ_{ST} for the introns was statistically significant (Mann-Whitney $U = 16$, $n = 9$, $P = 0.04$).

Four nonsynonymous amino acid substitutions were found in the three adult hemoglobin gene sequences. Two amino acid polymorphisms occurred in the αA subunit (Thr/Ala-$\alpha^{A}5$ and Ala/Thr-$\alpha^{A}8$), one was in the αD subunit (Met/Thr-$\alpha^{D}10$), and one was in the βA subunit (Asp/Glu-$\beta^{A}94$, Fig. 4.4). All four exhibited significant frequency differences between lowland and highland populations (Fig. 4.5). Pairwise F_{ST} values for these four nonsynonymous polymorphisms were 0.47, 0.07, 0.61, and 1.00, respectively.

As indicated by the F_{ST} values, *L. s. alticola* exhibited high levels of heterozygosity on the αA subunit. Excluding Mendoza, 31% were homozygous for Thr-$\alpha^{A}5$, 55% were heterozygous for Thr/Ala-$\alpha^{A}5$, and 14% were homozygous for Ala-$\alpha^{A}5$. In contrast, all except one *L. s. specularioides* were homozygous for Ala-$\alpha^{A}5$; this individual was heterozygous for Thr/Ala-$\alpha^{A}5$. Ala-$\alpha^{A}8$ was a rare allele found in only eleven *L. s. alticola* individuals collected in the highlands; all other individuals found in the lowlands and highlands were homozygous for Thr-$\alpha^{A}8$. Finally, the Thr-$\alpha^{A}5$ and Ala-$\alpha^{A}8$ alleles

do not occur on the same haplotype groups in crested ducks and probably evolved from divergent ancestral sequences (n = 80, Table 4.2). Based on a previous study of hemoglobin polymorphism in waterfowl, Thr-α^A5 and Ala-α^A8 were determined to be derived, whereas Ala-α^A5 and Thr-α^A8 are the ancestral amino acid residues found in other waterfowl species sampled worldwide (McCracken et al. 2009b).

Highland crested duck also exhibited high levels of heterozygosity on the αD subunit. Excluding Mendoza, 49% were homozygous for Met-α^D10, 39% were heterozygous for Met/Thr-α^D10, and 12% were homozygous for Thr-α^D10. All *L. s. specularioides* were homozygous for Thr-α^D10 (Fig. 4.5, Table 4.2).

For the βA subunit, highland and lowland crested ducks collected outside Mendoza were fixed for opposite alleles, Glu-β^A94 or Asp-β^A94, respectively (Fig. 4.5, Table 4.2). Within Mendoza, four of the eight individuals were heterozygous for Asp/Glu-β^A94, and two each were homozygous for Glu-β^A94 and Asp-β^A94, respectively (Table 4.2). Glu-β^A94 is the derived allele, and Asp-β^A94 is the ancestral allele found in other waterfowl species (McCracken et al. 2009b).

The three hemoglobin genes thus exhibited two distinctly different patterns. 100% of highland crested ducks were homozygous for Glu-β^A94, whereas most of them were heterozygous for Thr/Ala-α^A5 (55%) or Met/Thr-α^D10 (39%). In contrast, the same three derived alleles (Thr-α^A5, Met-α^D10, and Glu-β^A94) were rare or absent in the lowland population. Of the 81 possible combinations of alleles for each of the four amino acids substitutions found in the three adult hemoglobin subunits, 19 distinct genotypes were found. In the highlands every individual was homozygous for two or three

hemoglobin polymorphisms or homozygous for one polymorphism and heterozygous for two or more polymorphisms. In the lowlands, all but one individual possessed the ancestral genotype (Table 4.2). Only in Mendoza did we find unique combinations of genotypes that were not sampled in either of the other two habitats (Table 4.2). Seven different unique genotypes were observed in eight individuals in this region, and it was the only place in which Asp/Glu-$\beta^{A}94$ heterozygotes occurred. Thus, the highest level of heterozygosity was found in the Mendoza subsample.

When hemoglobins were added to the STRUCTURE analysis including the six introns, the classification, or assignment to either population, was improved notably (Fig. 4.3B). Excluding Mendoza, the mean probability of assigning *L. s. alticola* to the highland population was 0.99 ± 0.02 (range: 0.86–0.99), and the mean probability of assigning *L. s. specularioides* to the lowland population was 0.98 ± 0.03 (range: 0.83–0.99). This result was not an artifact of including more loci in the analysis, because the same results were observed as with the six introns alone when we randomly resampled three intron loci. The eight individuals collected at intermediate elevation in Mendoza (shown in grey in Fig. 4.3), as in the introns-only analysis, showed intermediate posterior probabilities compared to the other individuals (range = 0.09–0.98), consistent with a transition zone occurring over short geographic and elevational distances in southern Mendoza.

Finally, we tested Hardy-Weinberg (HW) equilibrium for the amino acid polymorphisms in the three globin genes. When highland and lowland populations were tested separately, all populations were found to be in HW equilibrium (*P*s > 0.05).

However, when highland and lowland populations were pooled, all HW tests were highly significant. More homozygotes and fewer heterozygotes were observed than expected in the three hemoglobin loci (Ps < 0.01).

4.4.3 COALESCENT ANALYSIS

4.4.3.1 Immigration rates between the highlands and the lowlands

The joint estimated migration rate for the six introns into the highlands (mH = 8.11; 90% HPD_{adj} = 1.24–20.46) exceeded the migration rate into the lowlands (mL = 1.29; 90% HPD_{adj} = 0.01–15.11; Table 4.3, Figure 4.6). Following divergence, the joint estimate for the six introns indicated that there were on average approximately 2.6 migrants/generation (m/G) into the highlands from the lowlands, and effectively no migration into the lowlands from the highlands ($4N_m$ = 0.3 m/G).

For the three globins, no migration was detected in any direction ($4N_m$ < 1), with the exception of the αA hemoglobin gene, which showed a small ($4N_m$ = 0.90) but non-zero estimate of migration into the highland population from the lowland population. In contrast to the introns, the lower bound of the posterior probabilities for the three globins overlapped zero in both directions (Table 4.3, Figure 4.6).

The same pattern as that seen for globins also occurred with mtDNA. No migration was detected in either direction, and the lower bound of the posterior distributions overlapped zero (Table 4.3).

4.4.3.2 Effective population size (N_e) and Theta (Θ) estimates

Based on joint Θ estimates for the six introns, we calculated an effective population size (N_e) of approximately 93,000 highland crested ducks (90% HPD_{adj} = 44,000–186,000 individuals) and 76,000 lowland crested ducks (90% HPD_{adj} = 29,000–183,000 individuals, Table 4.3). The N_e estimates for the highlands and lowlands were thus overlapping, and our estimates assume that lowland and highland crested ducks experienced similar substitution rates. The ancestral N_e was estimated to be around 41,000 individuals (90% HPD_{adj} = 500–900,000 individuals), suggesting that both populations have grown in size following divergence.

MtDNA estimates suggest an N_e of approximately 121,000 highland crested ducks (90% HPD_{adj} = 66,000–202,000 individuals) and 66,000 lowland crested ducks (90% HPD_{adj} = 28,000–146,000 individuals) with an ancestral N_e of 78,000 individuals (90% HPD_{adj} = 200–404,000 individuals). The N_e estimates from the mtDNA thus also overlapped the estimates from the introns.

N_e calculations for the globins are not useful, given that the apparent mutation rate is likely affected by selection. Although Θ 90% HPD_{adj} values were broadly overlapping between the highlands and the lowlands for HBA1, HBA2, and HBB, the mean was empirically higher in the lowlands for HBA1 and HBA2, and the pattern was reversed for HBB. Similarly, the mean Θ was also greater in the highland population than the lowland for the six introns and for mtDNA.

4.4.3.3 Time since divergence and TMRCA

The mean values for TMRCA ($t \times \mu$) ranged between 0.25–0.68 for the six introns, and ranked ENO1 > LMNA > GRIN1 > FBG > ODC1 > PCK1 (Table 4.3, Figure 4.7). The *TMRCA* values for the globins were 0.60 for HBA1, 0.86 for HBA2, and 2.58 for HBB, respectively. The mean TMRCA values for HBA2 and HBB thus, exceeded the introns. MtDNA had the deepest TMRCA of all loci: 5.62 (Table 4.3).

Based on the joint estimate for the six introns, time since divergence between highland and lowland populations was estimated to be 285,000 years ago. Nevertheless, the lower and upper bound of the 90% HPD_{adj} could not be estimated because the posterior distribution had a rising upper tail. Based on mtDNA, time since divergence was approximately 108,500 years ago (90% HPD_{adj} = 53,400–252,100).

4.4.3.4 Recombination

Most intron loci either lacked recombination or had too little polymorphism to allow recombination to be detected (ENO1, FGB, ODC1, and PCK1). The recombination rate (ρ/μ) for HBA1 was 0.7, and for HBA2 was 1.07. However, recombination rate estimates for LMNA (1.10) and GRIN1 (2.77) exceeded estimates for the hemoglobins. The lack of evidence of recombination for HBB was likely due to the fact that we analyzed only a portion of the whole gene sequence. HBB is recombining in this species (see McCracken et al. 2009a).

Finally, no evidence of gene conversion was found between the paralogous αA and αD subunits. The average percent amino acid sequence identity between HBA1 and HBA2 was 58.6%.

4.5 Discussion

4.5.1 CONTRASTING DIFFERENTIATION PATTERNS AND GENE FLOW

Analysis of multilocus genetic variation revealed a significant pattern of genetic differentiation between lowland and highland populations of crested ducks. The Φ_{ST} values for the six autosomal reference loci ranged from 0.02 to 0.44. Historical migration rates based on the joint estimate for the introns averaged 2.6 effective migrants/generation into the highlands from the lowlands, and the 90% confidence interval did not overlap zero. By contrast, effectively no migration was observed into the lowland population from highland birds following divergence.

The Φ_{ST} values for the three hemoglobin genes were generally greater (range 0.23–0.87), with the βA hemoglobin gene showing the greatest differentiation of any locus. Coalescent analyses for these three loci showed little evidence of allelic migration between highland and lowland populations, with the exception of the αA hemoglobin gene, which showed a small ($4N_m = 0.90$) but non-zero estimate of migration into the highland population from lowland birds.

Geographic analysis of gene flow thus documented a strong pattern of hemoglobin differentiation in the different environments that crested ducks inhabit. The mismatch in migration estimates between the globins and the autosomal introns (all

nuclear loci) likely reflect the less restricted movement of neutral nuclear alleles along the elevational gradients relative to the hemoglobin haplotypes. Four derived amino acid polymorphisms were found segregating at high frequencies in the highland population, whereas the lowland population was fixed for the ancestral hemoglobin alleles.

4.5.2 SIGNIFICANCE OF HEMOGLOBIN AMINO ACID REPLACEMENTS

Of the four amino acid replacements found in crested ducks, Glu-β^A94 in particular is potentially important in influencing hemoglobin O_2-binding affinity. Avian hemoglobins bind to allosteric effectors, such as IPP, in both oxy and deoxy forms, with a higher affinity in deoxy form (Rollema and Bauer 1979). Allosteric effectors lower Hb-O_2 affinity by strengthening salt bridges that favor the low-affinity T structure of hemoglobin (Perutz 1983). The positively charged groove at the entrance to the central cavity between the two β chains is a binding site of IPP (Fig. 4.4, Liang et al. 2001a). The Glu-β^A94 substitution lies adjacent to this binding region and IPP binding sites at β^A144 and β^A146 (Fig. 4.4, Liang et al. 2001a). In human hemoglobin, a salt-bridge between the N-terminal His-β^A146 and Asp-β^A94 stabilizes the low-affinity deoxy structure and decreases hemoglobin oxygen affinity (Perutz 1970, Shih et al. 1993). Bar-headed goose deoxy hemoglobin has been shown to lack this salt bridge, which weakens the Bohr effect and increases the hemoglobin O_2-affinity (Liang et al. 2001b). The presence of this salt bridge in crested duck is yet unknown. Furthermore, McCracken et al. (2009b) found that endemic highland populations of puna teal (*Anas puna*) are also fixed for Glu-β^A94, whereas lowland populations were fixed for the same ancestral residue Asp-β^A94 shared

by crested duck. Experiments examining sensitivity of Hb-O_2 binding to IPP and CO_2 and site-directed mutagenesis would help determine whether this substitution is adaptive in crested duck.

In contrast to the αA subunit, which possessed only a single amino acid replacement, two derived substitutions were observed on the A helix of the αA subunit of crested duck. McCracken et al. (2009b) found that highland populations of two other Andean waterfowl species also possessed derived amino acid substitutions in this same structural domain of the HbA tetramer: Andean goose (*Chloephaga melanoptera;* Ala-$\alpha^{A}8$) and cinnamon teal (*Anas cyanoptera*; Ser-$\alpha^{A}9$). Amino acid residues $\alpha^{A}5$, 8, and 9 each occupy external, solvent-accessible positions in the HbA structure (McCracken et al. 2010), and this region of the protein is known to undergo an important conformational change during the transition from the deoxy to the oxy state (Perutz 1990).

Finally, a fourth derived amino acid replacement was observed in crested duck occurring in the same structural domain of the HbD tetramer: Met-$\alpha^{D}10$ ($\alpha^{D}10$ corresponds to $\alpha^{A}11$ in HbD and HbA, respectively, due to one amino acid difference in the length of the αD and αA chain subunits). Interestingly, the endemic highland species Andean goose also possess a derived substitution in this region of the HbD protein (Leu-$\alpha^{D}9$, McCracken et al. 2010). Thus, the A helix of the αA and αD-chains is likely to be an important region of the hemoglobin protein involved in hypoxia resistance, warranting further study. However, little physiological data are available for these species, and oxygen dissociation curves for crested ducks have not been obtained, so the effects of Thr-$\alpha^{A}5$, Ala-$\alpha^{A}8$, Met-$\alpha^{D}10$, and Glu-$\beta^{A}94$ are yet to be determined unambiguously.

In sum, the general lack of evidence for gene flow for hemoglobin alleles between highland and lowland populations of crested ducks, as compared to the autosomal introns, and the biochemical properties of the amino acid substitutions themselves are consistent with the effects of selection acting on these loci. Selection exerted by different oxygen partial pressures at different elevations has been shown to be a strong determinant of hemoglobin haplotype frequencies in mice (Snyder 1981; Snyder et al. 1988; Storz et al. 2007). This is also likely to be the case in crested ducks. If highland hemoglobin alleles confer higher affinity for oxygen or decreased sensitivity to allosteric effectors such as IPP, then their effects are likely advantageous in the highlands and individuals that possess such alleles would be expected to have higher fitness.

4.5.3 HOMOZYGOSITY AND HETEROZYGOSITY IN HEMOGLOBIN GENES

One significant pattern we observed was that the high levels of heterozygosity in the duplicated α-globins contrasted sharply with the low heterozygosity found in the β subunit. Several possible factors might contribute to this pattern. It is possible that the amino acid polymorphism on the β subunits is an older replacement than the polymorphisms on either of the α subunits and that it has had more time to evolve to fixation. But if selection is strong, this may not be the case, as fixation could have proceeded rapidly. Another possibility is that different types of balancing selection may be operating on polymorphisms within different hemoglobin loci. A simple model of divergent selection with local adaptation favoring different homozygous genotypes appears to be consistent with the pattern observed for the βA subunit. In contrast, the

most common α-globin genotypes for the αA subunit were heterozygous in the highlands. Such patterns would not be inconsistent with a model of overdominance. Overdominance occurs when individuals that are heterozygous for certain alleles have superior fitness than that of the respective homozygotes. Heterozygous genotype classes can be favored when the environment is variable or fluctuating (Lewontin and Hubby 1966, Nielsen 2005) or if individuals seasonally occupy a range of different environments in which two or more different alleles each contribute to higher average fitness.

In the case of hemoglobin, heterozygous individuals would be expected to have a mixture of different Hb isoforms with potentially different O_2-binding properties. Such variation in physiochemical characteristics might very well enhance the flexibility of an organism living in a variable environment. The highest levels of heterozygosity we observed were in Mendoza, and crested ducks there are known to descend seasonally to lower elevations when too much snow accumulates in the cordilleras (Young 2005). Heterozygous individuals might have higher fitness if the different alleles result in codominant mixtures of hemoglobin with high, intermediate, and low affinity isoforms with distinct binding capability. Individuals with a wider range of hemoglobin function might have a dispersal advantage (~1,000 to 3,000 m). Additional sampling and physiological studies are required to evaluate and test these hypotheses.

Finally, McCracken et al. (2009a) also found elevated αA subunit heterozygosity in three out of four waterfowl taxa with amino acid replacements on both the αA and βA hemoglobin subunits. The major (HbA) and minor (HbD) bird hemoglobin isoforms differ only in composition of the α chains (αA vs. αD); the β chains are identical (Weber

et al. 1993). The erythrocytes of adult birds thus contain a mixture of functionally distinct hemoglobin isoforms with different biochemical properties (Hoffmann and Storz 2007). Therefore, it is possible that selection on the βA subunit might be more constrained than for the αA or αD subunits because the βA subunit occurs in two different isoforms with different biochemical properties and different oxygen-binding properties (Cirotto and Geraci 1975, Baumann et al. 1984, Riggs 1998, Knapp et al. 1999). This is consistent with Oberthür et al. (1983), who found that the avian β-globin chains have evolved more slowly, presenting a significantly lower apparent mutation rate than either of the α-chains. Oberthür et al. (1983) suggested that constrained evolution on the β-chains arose because of interactions with IPP in the hemoglobin central cavity.

4.5.4 ECOGEOGRAPHIC FEATURES OF POPULATION STRUCTURE

In our previous morphometric study (Bulgarella et al. 2007), we found evidence of geographic variation in morphology in crested ducks, with larger-bodied individuals at higher elevations in the Andes (3,000–4,600 m), smaller individuals inhabiting the coastal and inland lowlands of Patagonia, and Mendoza specimens being intermediate in size between the two populations. Based on ten morphological measurements and plumage, seven of the eight Mendoza individuals were classified as *L. s. alticola*, and only one female (KGM 1221) collected at 2,093 m in southern Mendoza was classified as *L. s. specularioides*.

Our results indicate that gene flow between the highland and lowland crested duck populations has been sharply restricted for hemoglobins and mtDNA, but some

measurable gene flow exists for the autosomal introns. Crested ducks are continuously distributed from central Peru through Tierra del Fuego. However, their densities decrease where the subspecies ranges are adjacent. Crested ducks are abundant in the arid puna/altiplano environment south to the province of Mendoza, Argentina (~ 1,500 m of elevation). The lowland subspecies is common from southern Chubut, Argentina, southwards throughout the Patagonian steppe (0–950 m). In the low elevation (< 1,500 m) Argentine provinces that lie between these regions, Neuquén and Río Negro, crested ducks are uncommon. Crested ducks thus occur at high densities in the altiplano and on steppe and coastal Patagonia environments, but at low densities through the relict conifer *Araucaria araucana* forest and the *Nothofagus* spp. forest that is widespread in southern Neuquén and Río Negro and throughout Chile.

Despite approximately 1,770 linear km between the southernmost collected Catamarca specimen (Laguna de los Aparejos, –27.6475°S) and the northernmost Chubut sample (Tecka, –43.6062°S), analyses for the six introns yielded non-zero estimates of gene flow from the lowlands into the highlands; and as noted in Methods, our coalescent analysis included individuals from the highland and lowland populations *per se* but excluded the individuals collected in the intermediate region of Mendoza. Thus, it is intriguing that gene flow was detected across this region given that the *Nothofagus* forest is mostly unsuitable habitat for the species, and no crested ducks were observed between Pino Hachado, Neuquén, and Tecka, Chubut in any of our expeditions (Bulgarella and McCracken pers. obs.).

Gene flow across the *Nothofagus* forest region could be facilitated through high elevation occupancy above timberline during the summer and in ice-free alpine lakes, or via the steppe and pre-Cordillera, particularly during winter. So while there are likely few or no crested ducks in the forest, there is abundant puna-like habitat in the pre-Cordillera and above the tree line in the talus slopes, making it possible for the ducks to inhabit this region. Nevertheless, caution is required when interpreting these results because present day distributions may not reflect past distribution patterns for the subspecies or the vegetational characteristics of the region.

MtDNA haplotypes transitioned from highland to lowland not in the province of Mendoza, as did hemoglobin haplotypes, but somewhere in the southern provinces of Neuquén or Río Negro at a lower elevation. This pattern was evident because in Mendoza we found that all individuals had 'highland' mtDNA haplotypes. The two individuals we collected in northern Mendoza (-32.8518°S) were morphologically similar to highland crested ducks and had 'highland' mtDNA haplotypes. Southern Mendoza birds (collected between -34.8457°S to -35.8162°S) had 'highland' mtDNA haplotypes, admixed nuclear haplotypes, and intermediate morphology, more similar to that of the individuals we collected in Patagonia. In sum, the pattern we observed for mtDNA differed from the patterns we observed based on morphology and examination of six autosomal nuclear introns that did not seem to have followed a pattern, and their transition does not correspond to a simple transition zone (Fig. 4.2). This mitonuclear discrepancy may possibly reflect the effects of genetic drift on a locus (mtDNA) with one quarter the effective population size than that of nuclear loci (Hudson 1990). An alternative

explanation is that our sample size in Mendoza (n = 8) is still not large enough to allow us to find shared mtDNA haplotypes in this region, if they indeed exist. Peters et al. (2005) stressed the importance of sampling enough loci and enough individuals to avoid drawing erroneous conclusions from insufficient data. Lastly, variation in mtDNA haplotypes may be tracking the vicariant forest biogeographic barrier. Additional sampling at low, intermediate, and high elevations in the provinces of Mendoza, Neuquén, and Río Negro would provide valuable information as to what extent the transition zone between the environments extends.

We found further discordance in gene flow estimates between nuclear loci and mtDNA. Whereas we obtained a non-zero estimate of migration from the lowlands to the highlands for the autosomal introns, no detectable migration was found for mtDNA; the lower 90% confidence interval overlapped zero. These results could be explained by sex-biased dispersal, which can be reflected in genetic markers that differ in their modes of inheritance (i.e., biparentally versus uniparentally). Waterfowl are unusual among birds in that females show greater philopatry to natal and breeding areas than do males (Rohwer and Anderson 1988), and sex-biased dispersal has frequently been invoked as a possible explanation for low levels of mtDNA haplotype sharing among geographic regions (Scribner et al. 2001, Kulikova 2005, Sonsthagen et al. 2009). Genetic data may be particularly informative for crested ducks because little data on demographics and movement are currently available. Most observational studies classify crested ducks as essentially resident (Phillips 1922–1926, Delacour 1954), or as partially migrant (Young 2005). Additionally, histological analysis has shown that male crested ducks possess fully

developed germinal epithelium during all seasons (Breucker et al. 1989). Breucker et al. (1989) confirmed their histological analysis with field observations that at all times of the year when birds were collected, ducklings were found. Furthermore, both female and male crested ducks are known to take care of their ducklings for prolonged periods (Buitron and Nuechterlein 1989), and the species has been reported to be monogamous (Weller 1968, 1972). Therefore, we do not know whether sex-biased dispersal occurs in crested ducks or if mtDNA reflects accurate gene flow measures for both sexes.

4.5.5 INTERMEDIATE ELEVATIONAL HABITATS IN MENDOZA

Considering the four hemoglobin amino acid replacements, we found seven different unique genotypes in eight crested ducks from Mendoza that were not sampled in either the lowlands or the highlands (Table 4.2). The transition in hemoglobin haplotypes from highland to lowland occurred in Mendoza, farther north compared to the transition for mtDNA haplotypes. In southern Mendoza (~ 1,500 m), the beginning of the low-elevation environment, hemoglobin haplotypes were mainly lowland in composition, suggesting that hemoglobin variation is primarily driven by elevation. Selection exerted by different oxygen partial pressures at different elevations has been shown to be a strong determinant of hemoglobin haplotype frequencies in mice (Snyder 1981; Snyder et al. 1988; Storz et al. 2007). Moreover, Mendoza was the only region where we sampled crested ducks that were heterozygous for Asp/Glu-β^{A}94. Introgression, persistent gene flow, or seasonal movements may all be involved in the high levels of heterozygosity found in this region.

Two primary classes of models predict the maintenance of hybrid zones between species/subspecies in continuous habitats (Barton and Hewitt 1985, Runck et al. 2009). The tension zone model (Key 1968, Barton and Hewitt 1985) argues that clines are maintained by a balance between dispersal and selection against hybrids. Because tension zones are not maintained by a response to local environmental conditions, they can move from place to place (Barton 1979). Alternatively, the bounded superiority model holds that hybrids are more fit than pure parental phenotypes in restricted regions where hybrid zones occur (Moore 1977, Runck et al. 2009). Populations that diverged in response to selection regimes exerted by their respective communities may produce hybrids that are not less adapted to the transitional habitat than are the parental phenotypes. Therefore, a stable hybrid population would be established in the ecotone. Selection gradients exerted by the distinctly integrated ecological communities on either side of the ecotone would prevent expansion of the hybrid zone; and reproductive isolation would not evolve because, where the opportunity to hybridize occurs, there is no selection against hybrids (Moore 1977). By definition, the bounded superiority model incorporates overdominance; selection maintains a stable equilibrium at each locality through, for example, heterozygote advantage given that heterozygotes are more fit in the intermediate habitat than either parental class.

The bounded superiority model seems to be a better fit for the patterns we observed in Mendoza. Unusually high levels of heterozygosity were observed for the three hemoglobin genes within this region, and other nuclear DNA loci were highly admixed. It is possible that heterozygous individuals within this region experience higher

fitness if the different hemoglobin alleles result in codominant mixtures of high-, intermediate-, and low-affinity isoforms that are suitable to a seasonally variable environment, such as changes in snow conditions that occur in this region. The bounded superiority model further predicts that introgressants and pure parentals do not overlap extensively, with the introgressants being self-sustaining and not the result of continual hybridization (Moore 1977). We do not know if this assumption holds true in crested ducks.

4.6 Conclusions and conservation implications

Mountain systems are generally regarded as hotspots of biodiversity (Lomolino 2001, Körner 2002). The Andean zone of South America has a higher diversity of plant and animal life than any other part of the world (Fjeldså and Krabbe 1990). Nevertheless, climate change and resource development are currently threatening the Andean landscape with unknown biodiversity consequences. Furthermore, along gradients of elevation, diversity reaches a peak at intermediate elevations along the cline. The peak appears to correspond closely with transition zones between lowland and montane regions (Brown 2001, Lomolino 2001). Importantly, the higher level of genetic intra-population diversity at medium altitudes than that at the low or high altitudes suggests that populations at intermediate altitudes should be targeted as a first priority in terms of their conservation (Yan et al. 2009), and also for further physiological studies because of the unique genotypic classes that were observed in this environment.

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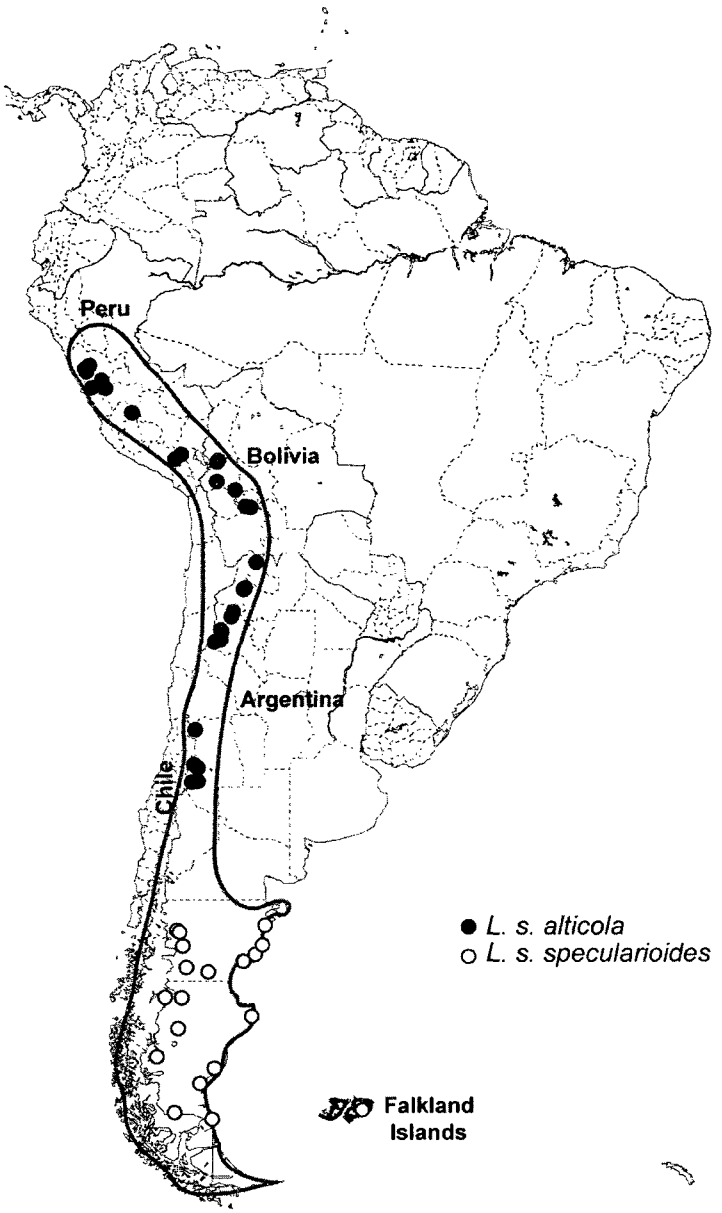

Figure 4.1 Geographic distribution and sampling locations of crested ducks in this study. The range for crested duck includes the coastal and inland habitats delimited by the contour line.

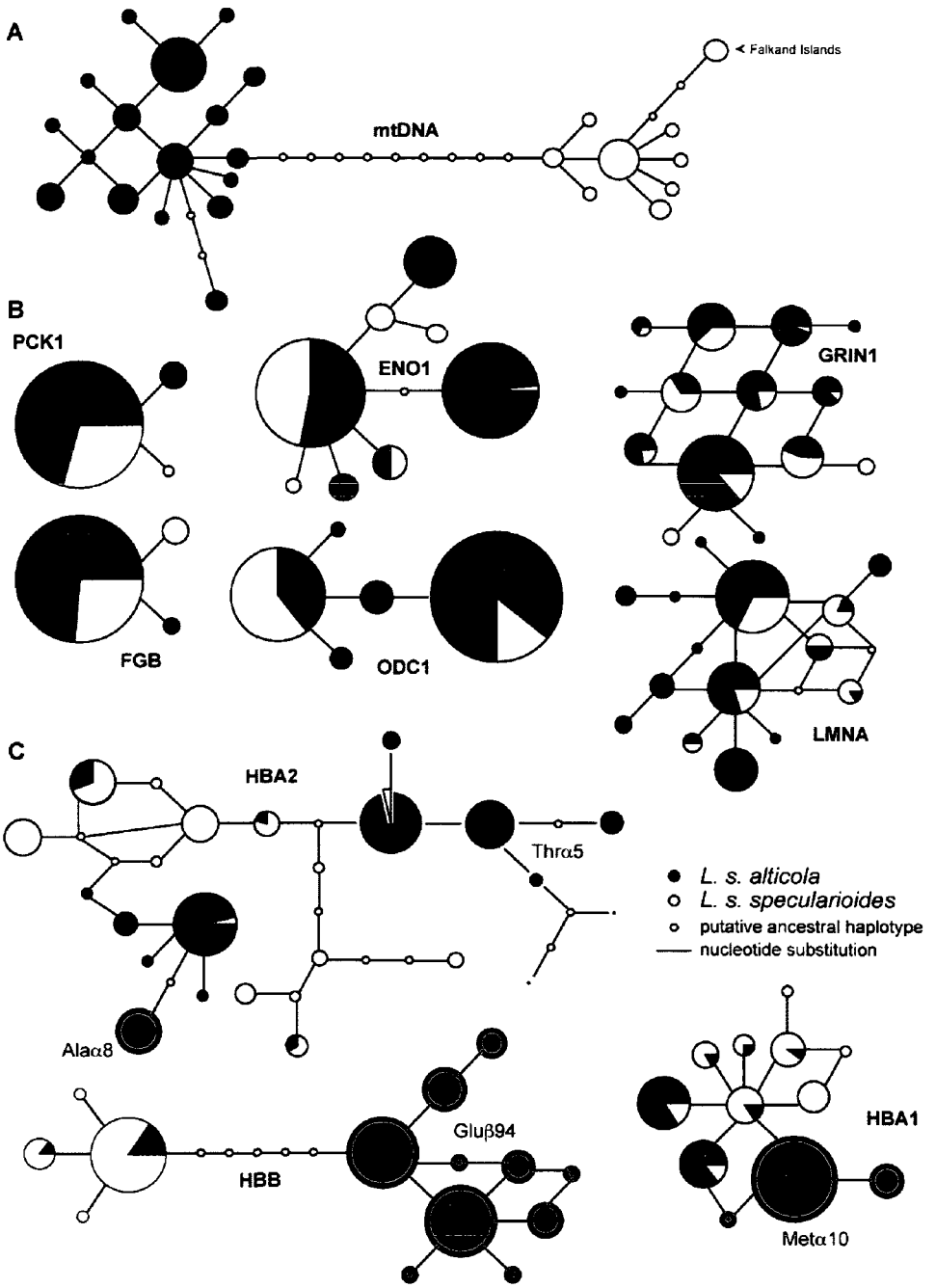

Figure 4.2 Unrooted parsimony networks showing the relationships among (A) mtDNA haplotypes, (B) the six introns, and (C) the three hemoglobin subunits. HBA1, HBA2, and HBB alleles possessing amino acids that occurred at high frequency in the highlands are shown with colored circles. The areas of the circles are proportional to the number of alleles found. The eight specimens from Mendoza were included in the networks (n = 80).

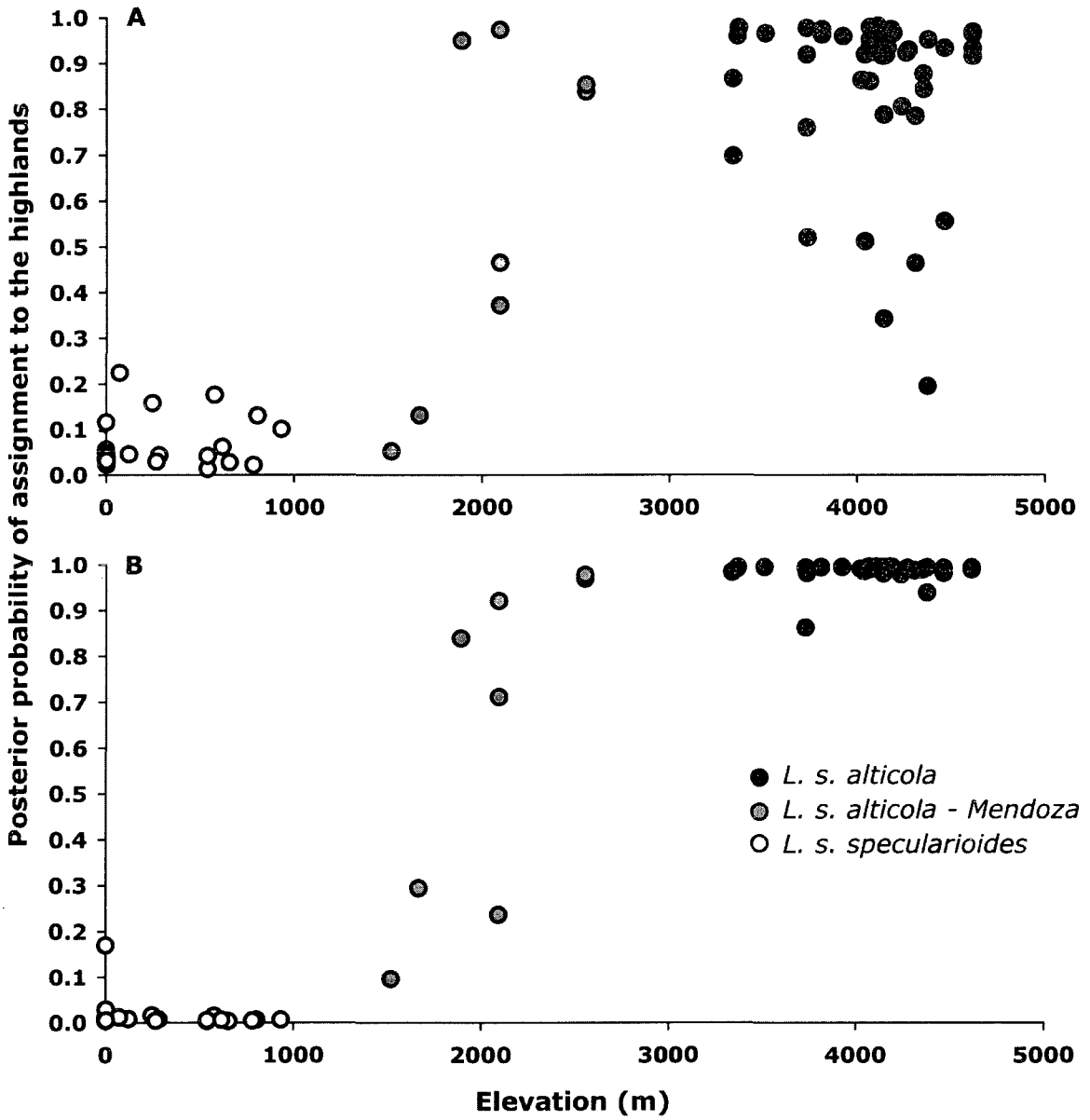

Figure 4.3 Posterior probability of assignment to the highland population versus elevation, based on Bayesian likelihood estimates for the 80 individuals in the study, for (A) the six autosomal introns, (B) the six autosomal introns and the three hemoglobin loci combined.

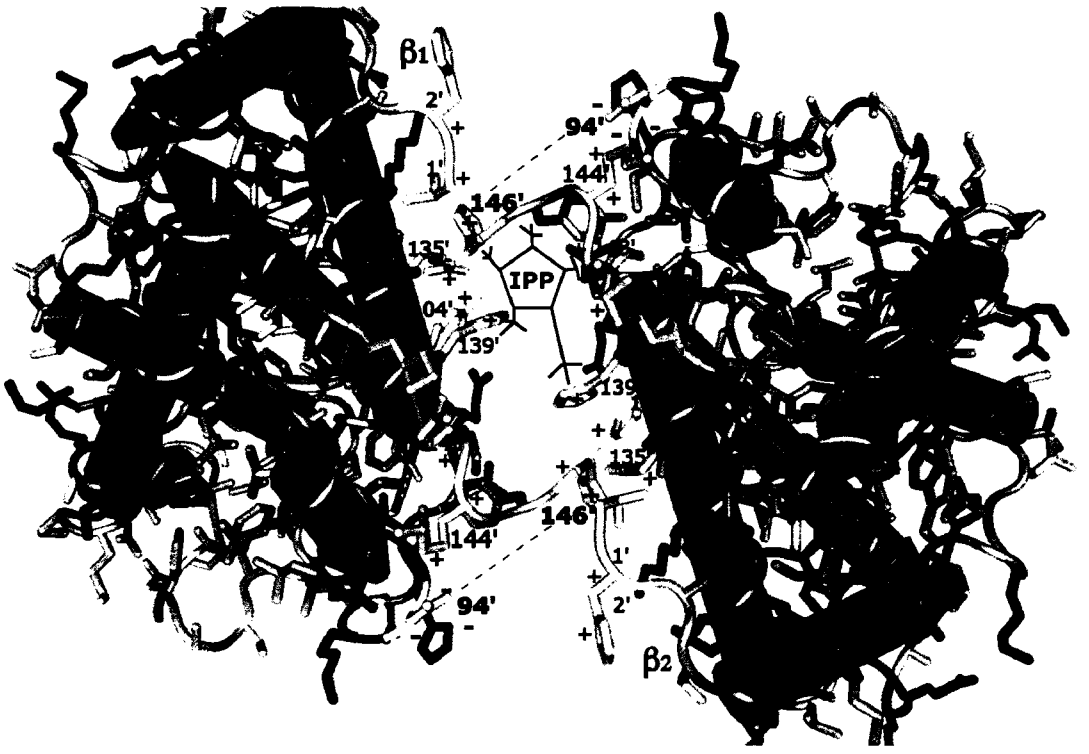

Figure 4.4 Binding of IPP in the central cavity between the β_1 and β_2 chains of deoxyHb. β94 and the 1st IPP binding sites β1, β2, β82, β104, β135, β139, β143, β144, β146 are shown in yellow. Also shown (in dashed lines) are intrachain salt-bridges formed in deoxyHb between the imidazole ring of the N-terminal β146His and the negatively charged β94Asp. This bond increases the affinity of β94Asp for protons, contributing to the Bohr effect.

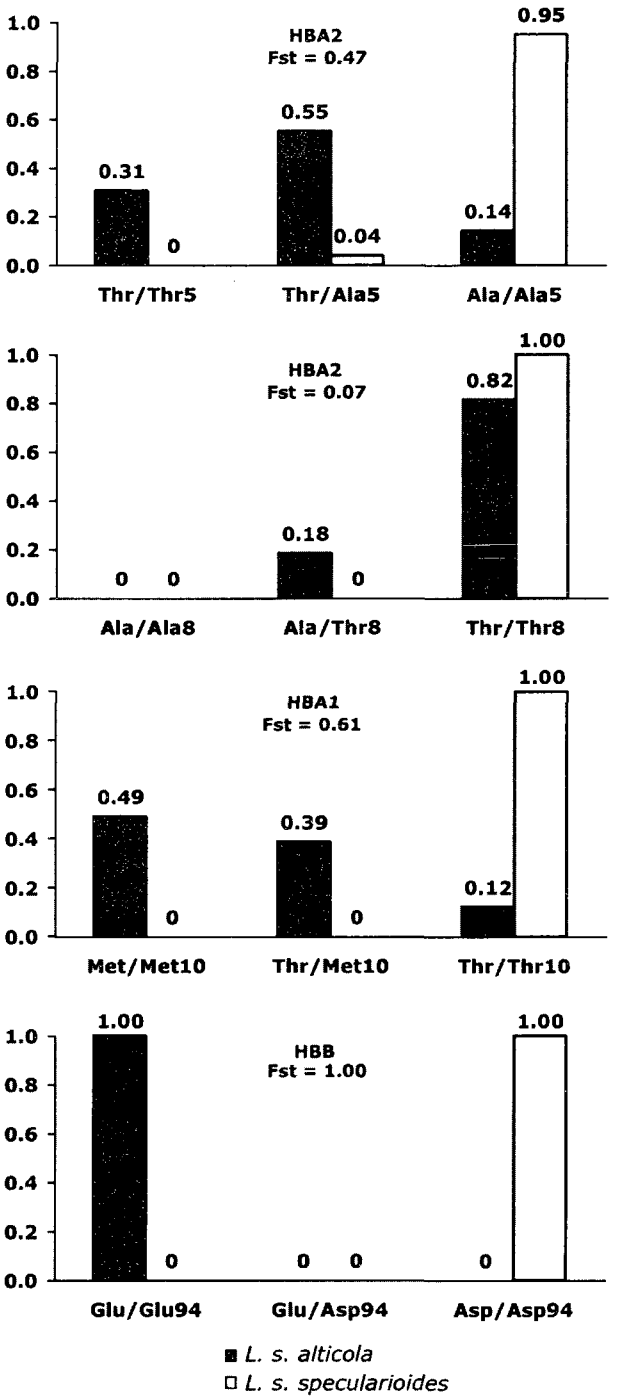

Figure 4.5 Allelic frequencies for four amino acid replacements observed in highland and lowland crested ducks hemoglobin subunits: Thr/Ala-$\alpha^{A}5$, Thr/Ala-$\alpha^{A}8$, Met/Thr-$\alpha^{D}10$ and Asp/Glu-$\beta^{A}94$.

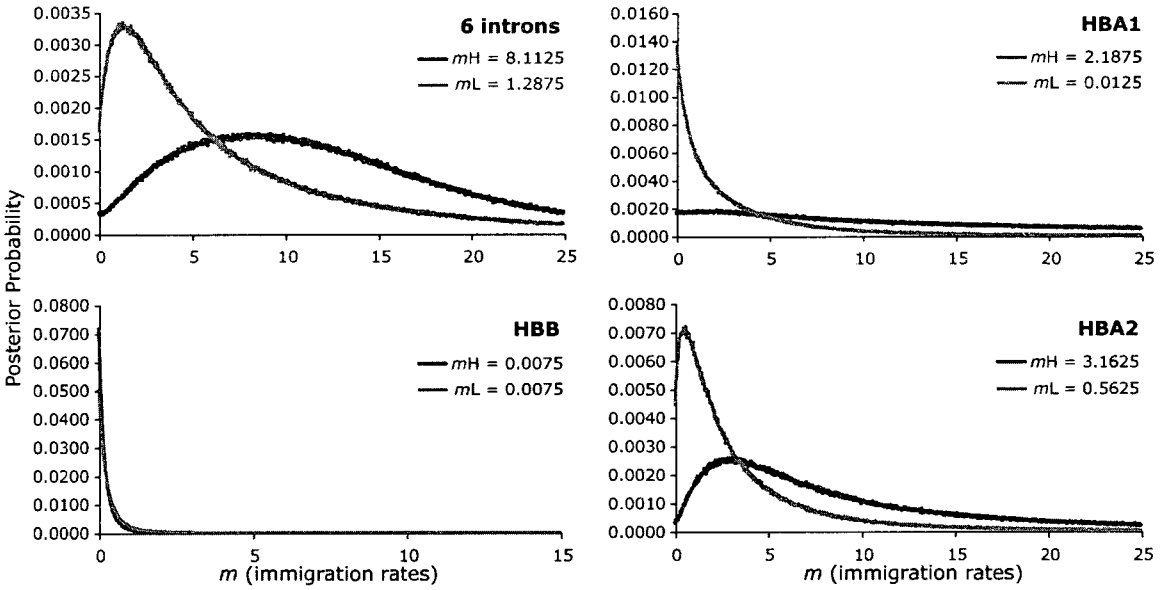

Figure 4.6 Migration rates into the highlands (*m*H), and into the lowlands (*m*L) for the six introns, and the three hemoglobin loci, respectively.

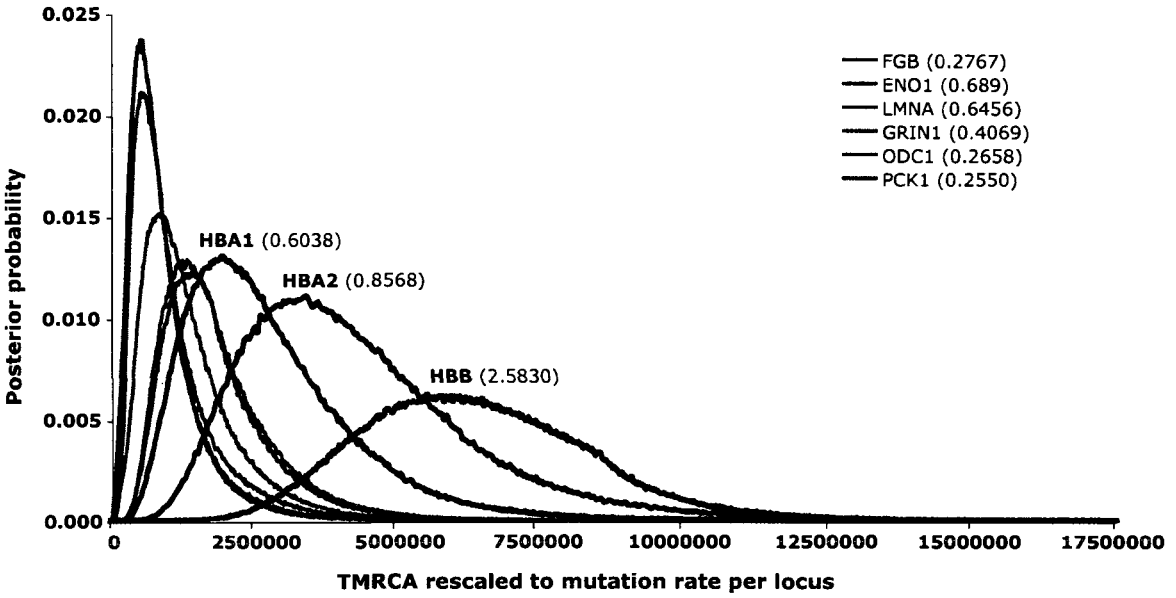

Figure 4.7 TMRCA ($t \times \mu$) estimates rescaled to the locus-specific mutation rate (μ), expressed in years before the present (YBP). The six introns are shown in grey and the three globins in color. The high point for each estimate is shown in parentheses for each locus.

Table 4.1 Population genetic parameter estimates for ten loci from lowland (n = 23) and highland (n = 49) populations of crested ducks.

Loci	Length (bp)	Chromosomal location[1]	No. alleles[2]	Allelic richness AR^2	Recombination rate (ρ/μ)	Φ_{ST3}
αD hemoglobin (HBA1)	465	14	**6**/9	**3.9**/8	0.70	**0.33**
αA hemoglobin (HBA2)	677	14	**12**/14	**8.6**/13	1.07	**0.23**
βA hemoglobin (HBB)	596	1	**9**/4	**6.3**/3	0	**0.87**
α enolase intron 8 (ENO1)	314	21	**5**/6	**3.2**/5	0	**0.21**
β fibrinogen intron 7 (FGB)	246	4	**2**/2	**0.7**/1	0	**0.13**
N-methyl D aspartate 1 glutamate receptor intron 11 (GRIN1)	335	9	**11**/11	**8.5**/10	2.77	0.02
Lamin A intron 3 (LMNA)	280	1	**15**/6	**10.1**/5	1.10	**0.10**
Ornithine decarboxylase intron 5 (ODC1)	353	3	**4**/2	**2.7**/1	0	**0.44**
Phosphoenolpyruvate carboxykinase intron 9 (PCK1)	345	20	**2**/2	**1**/1	0	0.03
mtDNA control region (mtDNA)	980	MT	**17**/9	**10.6**/8	–	**0.85**

[1]Based on the chicken genome (Hillier et al. 2004).
[2]Highland populations are shown in bold; eight individuals from Mendoza were excluded.
[3]Significant Φ_{ST} values are in bold ($P < 0.001$).

Table 4.2 The 19 genotypes found for each of the four amino acids substitutions in the three adult hemoglobin subunits of 80 crested ducks. Alleles observed at high frequency in the highlands are shown in bold.

HBA2 5	**HBA2** 8	**HBA1** 10	**HBB** 94	Highlands	Mendoza	Lowlands
Thr/Thr	Thr/Thr	**Met/Met**	**Glu/Glu**	3		
Thr/Thr	Thr/Thr	Met/Thr	**Glu/Glu**	6		
Thr/Thr	Thr/Thr	Thr/Thr	**Glu/Glu**	6		
Thr/Ala	Thr/Ala	**Met/Met**	**Glu/Glu**	2		
Thr/Ala	Thr/Ala	Met/Thr	**Glu/Glu**	5		
Thr/Ala	Thr/Thr	**Met/Met**	**Glu/Glu**	13		
Thr/Ala	Thr/Thr	Met/Thr	**Glu/Glu**	7		
Ala/Ala	Thr/Ala	**Met/Met**	**Glu/Glu**	3		
Ala/Ala	Thr/Ala	Met/Thr	**Glu/Glu**	1		
Ala/Ala	Thr/Thr	**Met/Met**	**Glu/Glu**	3		
Thr/Thr	Thr/Thr	Met/Thr	Asp/Asp		1	
Thr/Thr	Thr/Thr	Thr/Thr	Glu/Asp		1	
Thr/Thr	Thr/Thr	Thr/Thr	Asp/Asp		1	
Thr/Ala	Thr/Thr	Thr/Thr	**Glu/Glu**		1	
Ala/Ala	Thr/Thr	Thr/Thr	**Glu/Glu**		1	
Thr/Ala	Thr/Thr	Met/Thr	Glu/Asp		2	
Ala/Ala	Thr/Thr	Thr/Thr	Glu/Asp		1	
Ala/Ala	Thr/Thr	Thr/Thr	Asp/Asp			22
Thr/Ala	Thr/Thr	Thr/Thr	Asp/Asp			1
		Total number of individuals (n)		49	8	23

Table 4.3 IM estimates for the population size parameter theta ($\Theta = 4N_e\mu$), immigration rates ($M = m/\mu$), and TMRCA (t = TMRCA/μ) for six introns, three hemoglobin genes, and the mtDNA control region. The 90% upper and lower posterior parameter estimates are shown in parentheses.

Locus	$\Theta_{alticola}$	$\Theta_{specularioides}$	$\Theta_{ancestral}$	$m_{alticola}$	$m_{specularioides}$	TMRCA/μ (ybp) (IM TRMCA estimate)
Six introns	0.32 (0.15–0.63)	0.26 (0.10–0.63)	0.14 (0.00–3.08)	8.11 (1.24–20.46)	1.29 (0.01-15.11)	2,363,460 **ENO1** (0.6890) 577,763 **FBG** (0.2767) 2,995,129 **LMNA** (0.6456) 2,271,997 **GRIN1** (0.4069) 598,683 **ODC1** (0.2658) 1,715,825 **PCK1** (0.2550)
HBA1	0.05 (0.00–0.35)	0.79 (0.19–2.80)	---	2.19 (0.01–20.74)	0.01 (0.01–7.41)	1,969,545 (0.6038)
HBA2	0.29 (0.06–1.04)	0.63 (0.13–2.31)	---	3.16 (0.18–17.11)	0.56 (0.01–8.06)	3,415,066 (0.8568)
HBB	0.81 (0.29–1.89)	0.57 (0.13–1.68)	---	0.00 (0.00–0.55)	0.00 (0.00–0.82)	5,544,525 (2.5830)
mtDNA	18.31 (9.98–30.63)	9.98 (4.31–22.16)	---	0.00 (0.00–0.18)	0.00 (0.00–0.34)	119,494 (5.6210)

Appendix 4.1 Locality and specimen information for 80 crested ducks included in this study.

UAM no.	Field catalog no.	Date	Country	Province/ Department	Locality	Longitude	Latitude	Elevation (m)	Subspecies	Sex
	KGM-417	4 Oct 2001	Argentina	Salta	Abra del Gallo	-66.45360	-24.31390	4462	alticola	F
	KGM-418	4 Oct 2001	Argentina	Salta	Abra del Gallo	-66.45360	-24.31390	4462	alticola	M
	KGM-419	5 Oct 2001	Argentina	Salta	R. Corral Colorado	-66.56640	-24.39830	4135	alticola	M
17607	KGM-426	5 Oct 2001	Argentina	Salta	R. Corral Colorado	-66.58920	-24.41560	4081	alticola	M
17606	KGM-427	5 Oct 2001	Argentina	Salta	R. Corral Colorado	-66.58920	-24.41560	4081	alticola	F
	KGM-451	11 Oct 2001	Argentina	Jujuy	R. Corral Colorado	-66.71920	-22.71170	3510	alticola	M
19020	KGM-503	31 Oct 2001	Bolivia	La Paz	Río Desaguadero	-68.41670	-17.50000	3811	alticola	M
19021	KGM-504	31 Oct 2001	Bolivia	La Paz	Río Desaguadero	-68.41670	-17.50000	3811	alticola	M
19156	KGM-525	3 Nov 2001	Bolivia	Oruro	Lago Uru Uru	-67.14610	-18.03420	3735	alticola	M
19169	KGM-538	7 Nov 2001	Bolivia	Potosí	SE Ventilla	-66.09690	-19.17140	4119	alticola	M
19170	KGM-539	7 Nov 2001	Bolivia	Potosí	SE Ventilla	-66.09690	-19.17140	4119	alticola	F
19172	KGM-541	7 Nov 2001	Bolivia	Oruro	W Ventilla	-66.44970	-19.11250	4149	alticola	M
19173	KGM-542	7 Nov 2001	Bolivia	Oruro	W Ventilla	-66.44970	-19.11250	4149	alticola	F
19175	KGM-544	8 Nov 2001	Bolivia	Oruro	Lago Uru Uru	-67.14560	-18.03390	3730	alticola	M
19176	KGM-545	8 Nov 2001	Bolivia	Oruro	Lago Uru Uru	-67.14560	-18.03390	3730	alticola	F
19177	KGM-546	8 Nov 2001	Bolivia	Oruro	Lago Uru Uru	-67.14560	-18.03390	3730	alticola	M

22741	REW-094	11 Aug 2002	Peru	Junín	S Junín, Carretera Central, km 218
	REW-101	11 Aug 2002	Peru	Pasco	c. Cerro de Pasco
	REW-102	12 Aug 2002	Peru	Pasco	c. Cerro de Pasco, Carretera Central, km 294
	REW-104	12 Aug 2002	Peru	Pasco	c. Cerro de Pasco
20829	REW-107	12 Aug 2002	Peru	Pasco	c. Cerro de Pasco
	REW-130	23 Aug 2002	Peru	Ancash	Laguna Conococha
	REW-131	23 Aug 2002	Peru	Ancash	Laguna Conococha
	REW-139	25 Aug 2002	Peru	Ancash	Laguna Tapara, SE Pachacoto
	REW-140	25 Aug 2002	Peru	Ancash	Laguna Tapara, SE Pachacoto
	REW-160	30 Aug 2002	Peru	Ancash	Laguna Canrash
20793	REW-213	5 Oct 2002	Peru	Ayacucho	Razuhuilca, c. Huanta
	REW-214	6 Oct 2002	Peru	Ayacucho	Razuhuilca
	REW-215	6 Oct 2002	Peru	Ayacucho	Razuhuilca
20784	REW-289	21 Oct 2002	Peru	Puno	Laguna Lagunillas
20789	REW-290	23 Oct 2002	Peru	Arequipa	c. Imata
	REW-350	9 Dec 2002	Falkland Islands	East Falkland	Bertha's Beach, Fitzroy Farm
	REW-384	28 Dec 2002	Falkland Islands	East Falkland	Bertha's Beach, Fitzroy Farm
	REW-393	31 Dec 2002	Falkland Islands	East Falkland	Fitzroy, Fox Point

-75.94180	-11.23270	4177	alticola	F
-76.23740	-10.71240	4351	alticola	F
-76.23580	-10.71310	4257	alticola	F
-76.89150	-11.20410	4234	alticola	M
-76.22320	-10.69380	4188	alticola	M
-77.28350	-10.11970	4039	alticola	M
-77.28350	-10.11970	4039	alticola	F
-77.36490	-9.90790	4065	alticola	F
-77.36490	-9.90790	4065	alticola	M
-77.05580	-9.68520	4270	alticola	M
-74.17310	-12.87980	4100	alticola	M
-74.15460	-12.91380	4065	alticola	F
-74.15460	-12.91380	4065	alticola	F
-70.81310	-15.69090	4157	alticola	M
-71.24380	-15.97340	4349	alticola	M
-58.38380	-51.89090	3	specularioides	?
-58.38380	-51.89090	3	specularioides	?
-58.38380	-51.89090	3	specularioides	F

19628	KGM-719	20 Oct 2003	Argentina	Chubut	RP 17, W Tecka
19632	KGM-720	20 Oct 2003	Argentina	Chubut	RN 40, S Tecka
19626	KGM-726	22 Oct 2003	Argentina	Chubut	RN 40, W Shaman
19629	KGM-732	23 Oct 2003	Argentina	Chubut	RN 40, N Río Mayo
22747	KGM-746	26 Oct 2003	Argentina	Santa Cruz	RP 41, Estancia La Frontera
19636	KGM-749	26 Oct 2003	Argentina	Santa Cruz	RN 40, c. Estancias Telken & La Paloma
20781	KGM-753	28 Oct 2003	Argentina	Santa Cruz	RN 40, N Las Horquetas
19627	KGM-754	28 Oct 2003	Argentina	Santa Cruz	RN 40, N Las Horquetas
19630	KGM-774	31 Oct 2003	Argentina	Santa Cruz	Estancia Santa Margarita, c. Lago Viedma
19625	KGM-794	3 Nov 2003	Argentina	Santa Cruz	RN 40, c. El Zurdo
19640	KGM-795	5 Nov 2003	Argentina	Santa Cruz	RN 40, c. Estancia Monte Dinero
19631	KGM-802	6 Nov 2003	Argentina	Santa Cruz	RN 3, c. Paraje Lemarchand
19633	KGM-803	6 Nov 2003	Argentina	Santa Cruz	RP 288, c. Puerto Punta Quilla
19635	KGM-806	8 Nov 2003	Argentina	Santa Cruz	Bahía Río Deseado
19747	KGM-809	10 Nov 2003	Argentina	Chubut	S Lago Colhué Huapí
19637	KGM-820	11 Nov 2003	Argentina	Chubut	Bahía Bustamante
19634	KGM-821	11 Nov 2003	Argentina	Chubut	Bahía Bustamante
19639	KGM-824	12 Nov 2003	Argentina	Chubut	S Camarones

-71.06760	-43.60620	804	specularioides	F
-70.87550	-43.71010	934	specularioides	M
-70.67430	-44.38960	655	specularioides	M
-70.43980	-45.42210	578	specularioides	M
-71.86200	-46.84210	783	specularioides	M
-70.74550	-46.87610	618	specularioides	M
-70.97490	-48.30230	540	specularioides	F
-70.97490	-48.30230	540	specularioides	M
-72.41400	-49.55810	246	specularioides	F
-71.22580	-51.99600	122	specularioides	M
-68.66560	-52.26760	72	specularioides	M
-69.48180	-50.75020	281	specularioides	F
-68.48800	-50.08890	3	specularioides	M
-65.97270	-47.74210	0	specularioides	M
-68.94000	-45.65240	267	specularioides	F
-66.53500	-45.13480	0	specularioides	F
-66.52120	-45.14930	0	specularioides	M
-65.71630	-44.80330	0	specularioides	M

19624	KGM-827	13 Nov 2003	Argentina	Chubut	Cabo Raso
19638	KGM-828	13 Nov 2003	Argentina	Chubut	Playa Bonita, S Rawson
22749	KGM-1073	4 Nov 2005	Argentina	Catamarca	Laguna Antofagasta, Antofagasta de la Sierra
22744	KGM-1074	4 Nov 2005	Argentina	Catamarca	Laguna Antofagasta, Antofagasta de la Sierra
22739	KGM-1087	7 Nov 2005	Argentina	Catamarca	Río Punilla, 35 km N Antofagasta de la Sierra
22738	KGM-1088	7 Nov 2005	Argentina	Catamarca	Río Punilla, 35 km N Antofagasta de la Sierra
22743	KGM-1122	12 Nov 2005	Argentina	Catamarca	Río Chaschuil, S La Gruta
22745	KGM-1139	13 Nov 2005	Argentina	Catamarca	Río Chaschuil, c. Embalse Cortaderas
22748	KGM-1140	14 Nov 2005	Argentina	Catamarca	Río Chaschuil, c. Embalse Cortaderas
22735	KGM-1159	15 Nov 2005	Argentina	Catamarca	Laguna de los Aparejos
22751	KGM-1160	15 Nov 2005	Argentina	Catamarca	Laguna de los Aparejos
22737	KGM-1184	17 Nov 2005	Argentina	Catamarca	La Gruta
22742	KGM-1211	29 Nov 2005	Argentina	Mendoza	E Los Penitentes
22746	KGM-1212	29 Nov 2005	Argentina	Mendoza	E Los Penitentes
22734	KGM-1218	2 Dec 2005	Argentina	Mendoza	NW El Sosneado
22740	KGM-1220	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado

-65.23010	-44.33410	0	specularioides	M
-65.04820	-43.36090	0	specularioides	M
-67.42409	-26.11280	3338	alticola	M
-67.42409	-26.11280	3338	alticola	F
-67.28391	-25.82775	4140	alticola	F
-67.28391	-25.82775	4140	alticola	M
-68.06677	-27.02894	3923	alticola	F
-68.14524	-27.56000	3363	alticola	F
-68.14498	-27.55590	3369	alticola	F
-68.54215	-27.64755	4106	alticola	M
-68.54215	-27.64755	4106	alticola	F
-68.14566	-26.92542	4020	alticola	M
-69.80995	-32.85187	2552	alticola	F
-69.80995	-32.85187	2552	alticola	M
-69.63432	-35.01203	1670	alticola	M
-69.91977	-34.84570	2093	alticola	F

23413	KGM-1221	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado
22733	KGM-1224	2 Dec 2005	Argentina	Mendoza	Laguna El Sosneado
22736	KGM-1228	3 Dec 2005	Argentina	Mendoza	Pampa del Rodeo, 45 km SW Malargüe, RN 40
22750	KGM-1232	4 Dec 2005	Argentina	Mendoza	Río Grande
23412	REW-709	27 Nov 2005	Bolivia	La Paz	Laguna Khara Kkota
23415	REW-710	27 Nov 2005	Bolivia	La Paz	Laguna Khara Kkota
23411	REW-714	27 Nov 2005	Bolivia	La Paz	Laguna Kkota
23416	REW-715	27 Nov 2005	Bolivia	La Paz	Laguna Kkota
23419	REW-721	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23418	REW-723	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23417	REW-724	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota
23414	REW-727	27 Nov 2005	Bolivia	La Paz	Laguna Janyuo Kkota

-69.91977	-34.84570	2093	alticola	F
-69.91977	-34.84570	2093	alticola	M
-69.62811	-35.76321	1891	alticola	M
-70.06362	-35.81625	1522	alticola	M
-68.38254	-16.18806	4307	alticola	M
-68.38254	-16.18806	4307	alticola	F
-68.35228	-16.12941	4374	alticola	M
-68.35228	-16.12941	4374	alticola	F
-68.31933	-16.08057	4611	alticola	F
-68.31933	-16.08057	4611	alticola	M
-68.31933	-16.08057	4611	alticola	F
-68.31933	-16.08057	4611	alticola	M

General Conclusions

The research presented in this dissertation focused on adaptation to life in high-altitude environments. One of the most noticeable effects is hypoxia. The partial pressure of oxygen decreases with elevation by approximately 10% per 1,000 m. At 4,000 m, e.g., in the Andes or on the Himalayan plateau, the partial pressure of oxygen of inspired air is 60% of that at sea level (Beall 2007). Animals vary in their capabilities to withstand hypobaric hypoxia. Among vertebrates, birds are particularly well adapted for maintaining oxygen supplies to the tissues. Several recent studies have shown that genetically based adaptations that increase the O_2-binding properties of hemoglobins play an important role in physiological adaptation to high-elevation hypoxia (Storz et al. 2007, Weber 2007, Storz and Moriyama 2008).

In chapter 1, I performed a multi-locus phylogenetic analysis to determine the relationships of *Lophonetta* within the South American duck clade. Based on molecular data, Johnson and Sorenson (1998, 1999) found that *Amazonetta*, *Lophonetta*, *Speculanas*, and *Tachyeres* formed a monophyletic group. This finding was surprising given that *Tachyeres* had never before been related to the other three genera, the species vary strikingly in their morphology, and the fact that *Amazonetta* lives in complete allopatry in relation to the other three genera. The monophyly of the four South American duck genera and their relationship to the dabbling ducks were both strongly supported by a Bayesian analysis of eight mtDNA and nuclear loci for one individual each of *Amazonetta*, *Lophonetta*, *Speculanas*, *Tachyeres*, five representative *Anas* dabbling duck species, and 18 other waterfowl genera. However, relationships within this well-

supported clade were not fully resolved despite sequencing more than 10,000 characters from six independent linkage groups. The lack of resolution likely resulted from high levels of homoplasy and a lack of informative characters (i.e., soft polytomies), rapid divergence times among genera and species (i.e., hard polytomies), or a combination of these factors. Nevertheless, it is clear that this group underwent at least two periods of rapid diversification, one producing the four genera and a more recent radiation among the four *Tachyeres* species.

In chapter 2, I analyzed morphological differences between the two crested duck subspecies that inhabit different elevational environments. Specifically, I sought evidence for Bergmann and Allen's ecogeographic rules. Body size of crested ducks differed between the subspecies and between the sexes. *L. s. alticola* from the central high Andes found at 3,338–4,611 m were larger than *L. s. specularioides* from southern Patagonia (< 934 m to sea level). *L. s. alticola* individuals of intermediate body size were found at mid-elevations (1,522–2,552 m, Mendoza, Argentina). Crested ducks conform to Bergmann's Rule. No evidence was found for Allen's Rule. Intermediate-sized crested ducks, such as those found in Mendoza, might result from introgression between *L. s. alticola* and *L. s. specularioides*, and/or natural selection on body size of individuals locally adapted to intermediate elevational habitats.

Chapter 3 was based on the creation of a cDNA library from bone marrow of six crested ducks inhabiting the high Andes of Peru. The results obtained provide the first quantitative identification of gene expression in bone marrow of birds inhabiting high-altitude regions and are in agreement with the known hemopoietic and immune function

of this tissue. mRNA coding for the three subunits of adult hemoglobin proteins were among the five most common transcripts in the expression profile. Furthermore, 70% of the total level of α hemoglobin expressed corresponded to the αA subunit, and 30% to the αD subunit, in accordance with the known major and minor HbA and HbD components of bird blood (Borgese and Bertles 1965, Saha and Ghosh 1965).

In Chapter 4, I focused on the population genetic structure of highland and lowland populations of crested ducks. Four unique amino acid replacements were found in the globins that differed in frequency between highland and lowland populations with significantly elevated F_{ST} values. Coalescent analyses suggested that gene flow is restricted for hemoglobin alleles and mtDNA between highland and lowland populations, whereas some level of gene flow was detected for the introns. Divergent selection with local adaptation favoring different homozygous genotypes is consistent with the pattern observed for the single polymorphism found on the βA subunit. Overdominance likely shaped the evolution of high-altitude function in the α subunits. The eight individuals collected at intermediate elevations in Mendoza revealed unique combinations of hemoglobin genotypes not found elsewhere, intermediate morphology, and admixed nuclear DNA. A bounded hybrid-superiority model may explain the pattern and frequency of genotypes we observed in this region.

Overall, results from this dissertation provide information for different aspects of biological science: the natural history of *Lophonetta*, ecogeographic variation, gene expression in non-model organisms, the use of independent molecular markers with

different modes of inheritance and mutation rates to infer population structure, and the effects of selection on candidate genes known to function in high-altitude respiration.

The information provided here is also relevant for the conservation of these endemic duck populations. There is a high diversity of plant and animal life in the Andes (Fjeldså and Krabbe 1990), and climate change and resource development are currently threatening the Andean landscape with unknown biodiversity consequences. Highland endemic aquatic species will likely be among the first influenced by the effects of environmental changes. Understanding the traits and mechanisms that underlie local adaptation to high-elevation environments is imperative for the conservation of these species and their habitats.

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